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THE COMPOSITION OF THE LE AND HEMATOXYLIN BODIES OF SYSTEMIC LUPUS ERYTHEMATOSUS *

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The hematoxylin stained bodies of systemic lupus erythematosus which were recognized by Gross,[1] and Ginzler and Fox[2] were correctly believed by these authors to be derived from altered nuclei. So unique to systemic lupus are the changes leading to the formation of these bodies and aggregates that they have been regarded as pathognomonic of this disease, and serve as additional criteria for its anatomic diagnosis. With the discovery of the LE cell phenomenon[3-6] it became obvious that the hematoxylin bodies were the counterparts in the tissues of the LE bodies formed *in vitro*. Histochemical characterization of these bodies,[7,8] which appeared to indicate that they contained partially depolymerized deoxyribonucleic acid (DNA), led to the concept that a depolymerase (DNASE) was activated in systemic lupus.[7,9-13] However, serum levels of DNASE were not found to be significantly elevated in this disease,[11,14] and it was hypothesized[11-13] that depolymerization of DNA in the LE cell was due to release of an intracellular DNASE from an intracellular inhibitor of DNASE by an action initiated by the circulating LE factor of the blood. It has since been proposed that the LE factor itself permits entrance of serum protease into the cytoplasm which in turn releases the intracellular deoxyribonuclease from its inhibitor.[13,15]

While most interest centered about the state of DNA in the hematoxylin body, it was understood by Klemperer,[9] and Gueft and Laufer[10] that the body was a nucleoprotein complex, possibly containing still other substances. The importance of the protein moiety was emphasized

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by the suggestion of Gueft and Laufer[10] that the fibrinoid substance in systemic lupus erythematosus was the residue of degraded nucleoprotein of the hematoxylin bodies from which some or all of the stainable DNA had disappeared.

Many of our conceptions about the pathogenesis of the tissue changes occurring in systemic lupus erythematosus have thus depended upon histochemical analysis of the hematoxylin bodies. In the work of Klemperer and co-workers,[7] the state of nucleic acid in the hematoxylin bodies was inferred from microspectrophotometric measurement of the relative degrees of Feulgen reaction and methyl green staining. Interpretation of methyl green staining of DNA has since been somewhat complicated by the demonstration that it is influenced by factors other than the degree of "polymerization" of DNA, particularly by protein interference. The protein component of the hematoxylin body has barely been characterized thus far, but it was regarded primarily as a residue of nuclear origin by Klemperer,[9] and by Gueft and Laufer.[10]

The recent elaboration of specific and quantitative staining methods for proteins has provided additional means for studying the composition of the hematoxylin and LE bodies. Their composition was therefore reinvestigated with the objects of assessing the cause of the previously observed depression of methyl green binding by DNA in the hematoxylin bodies, and of gaining additional information on their protein components.

Materials and Methods

Tissues from 3 fatal cases of systemic lupus erythematosus were available for study.* Sections of kidney tissue from one of these, fixed in Carnoy's fluid, were used for microspectrophotometric measurement. The remaining tissues, which had been fixed in formalin, were washed to remove unbound formaldehyde, and were examined after successive procedures. Abundant LE bodies (the free nonphagocytosed material) were obtained from LE preparations made according to the "ring" method of Snapper and Nathan,[16] the method of Davis and Eisenstein[17] in which dried leukocytes, concentrated in buffy coat, serve as substrate cells, and the method of Lee,[18] in which atabrine (quinacrine) is used to prepare leukocytes for the action of the LE factor of the serum (Figs. 10 and 11). Smears were then fixed in methanol or in 10 per cent neutral buffered formalin. Sections stained with hematoxylin and eosin, or smears of LE preparations stained with Wright's solution were photographed or mapped in order to permit

* Tissue for study was generously contributed by Dr. Henry Michelson of the University of Minnesota, and Dr. Harlan Firminger of the University of Kansas.

reidentification of the same objects in all subsequent procedures. Slides were then destained in 70 per cent ethyl alcohol acidified with trichloroacetic acid prior to the application of other methods.

Methyl green staining was performed in a phenol-glycerine solution at pH 4.2, according to the specifications of Pollister and Leuchtenberger.[19] The sections were then treated in one of two ways. In one group, after removal of the methyl green with alcohol, the free basic groups of the protein were blocked by acetylation in pure acetic anhydride for two hours at room temperature. They were then restained with methyl green and the areas previously studied were rephotographed or remeasured. The conventional Feulgen technique was then carried out on some of these preparations. After removing the methyl green, the other group of slides was stained with a modified Feulgen reaction method for DNA[20] in which one normal trichloroacetic acid replaced hydrochloric acid in the hydrolysis and in the preparation of the Schiff reagent. After rephotographing or remeasuring the same fields, the nucleic acids were extracted with 5 per cent trichloroacetic acid at 90° C. for 15 minutes. The slides were then stained for histone with the anionic dye fast green FCF at pH 8.0-8.2, according to the directions of Alfert and Geschwind.[21] Quantitative cytophotometric determinations of "total" protein were made on free LE bodies in LE preparations, by means of the binding of naphthol yellow S (C.I. 10) (flavianic acid) to protein basic groups, as elaborated by Deitch,[22] and the cytochemical Millon reaction of Pollister and Ris[23] as modified by Rasch and Swift.[24] The Sakaguchi reaction for arginine, modified to yield a more stable color by rapid dehydration after reaction in an alkalinized alphanaphthol and hypochlorite mixture, was used to detect arginine residues. The utility of the Sakaguchi reaction for cytophotometry has been established by McLeish and co-workers.[25]

A microspectrophotometric apparatus[26] incorporating some of the modifications proposed by Pollister[27] and Moses[28] was employed with a tungsten ribbon-filament light source powered from a battery-buffered system designed to improve voltage stability. Radiation of desired wavelength was isolated with a Bausch and Lomb diffraction grating monochromator. The methyl green color was measured at 633 mμ, the Feulgen complex at 568 mμ, naphthol yellow S at 435 mμ, the fast green at 625 mμ, the Millon complex at 490 mμ, and the Sakaguchi reaction at 510 mμ. A constant plug of 3.5 μ diameter was circumscribed for photometric measurement of LE bodies, and a plug of six tenths or less of the short diameter of whole nuclei in sections. Measured values are reported in arbitrary units of apparent amounts or

concentrations of dye per body or nucleus. Amounts were calculated by multiplying extinction by area in the case of LE bodies, and extinction by $\frac{D^2}{F} \times \frac{d_b}{d_a}$ in the case of nuclei in sections, where D is the plug diameter, F the fraction of the nuclear volume included in the plug, and $\frac{d_b}{d_a}$ the ratio of the longer to the shorter diameter of the nucleus. The units of any set of determinations are not necessarily comparable with those obtained in other series of measurements; ratios, however, can always be compared from experiment to experiment.

Observations

Nucleic Acids

Deoxyribonucleic acid, which is principally responsible for the basophilia of cell nuclei, is among the most stable of the nuclear components. It is cytochemically demonstrable by its marked absorption of ultraviolet light at about 2,600 Å, by the Feulgen reaction given by its deoxypentoses, and by the affinity of its phosphate groups for basic dyes.

Methyl Green Staining

Although most basic dyes exhibit no such specificity of staining, the cationic dye methyl green has been known to cytologists for many years as a selective and reproducible nuclear stain.[19] It has been shown that under certain conditions, methyl green (heptamethylpararosanilin) can combine selectively and even stoichiometrically with available phosphoryl groups of polymerized DNA[19,29-31] and it has therefore been employed for cytophotometric measurement of DNA in nuclei.[19,32-35] Depolymerization of DNA was found to reduce its binding of methyl green *in vitro*.[29,36] This fact was thought to explain the microspectrophotometric depression of methyl green stainability observed in cytologic material under various conditions,[19,33,35-38] assuming intracellular alteration of DNA "in the nature of depolymerization."[7] Depression of methyl green binding by hematoxylin bodies of systemic lupus erythematosus as compared with normal nuclei was thus interpreted to mean that in the transformation of nuclei in this disease, DNA became depolymerized.[7-10] However, it is known that various factors, including the presence of cations,[30,31,39] and especially associated proteins, effectively interfere with methyl green binding by nucleic acids.[40] Methyl green appears to be more sensitive to such interference than other basic dyes.[40] The influence of these factors must be accounted for before it is possible to assess cytochemically the state of polymerization of molecular configuration of DNA in tissues.

To evaluate the possible effect of protein interference on the methyl green binding by DNA in hematoxylin and in LE bodies, the methyl green staining in the same bodies was photographed or measured microspectrophotometrically before and after acetylation of protein basic groups. Acetylation is one of the ways of blocking the positively charged groups of proteins which compete with basic dyes for the available anionic binding sites of the nucleic acid.[22,40-42] Figures 6, 7, 12 and 13 illustrate some intensification of methyl green staining of hematoxylin and LE bodies after acetylation.

Microspectrophotometric comparison of the amounts of DNA revealed by methyl green in methanol-fixed lymphocytes and LE bodies in an atabrine LE preparation,[18] before and after acetylation, is shown in Table I. After acetylation of control lymphocytes, the amount of methyl green bound to DNA is augmented by about 6 per cent. In lupus bodies, however, blocking of protein basic groups by acetylation

TABLE I

Mean Amounts of DNA in Arbitrary Units as Determined by Methyl Green Binding and the Feulgen Technique

	Blood		Kidney	
	Lymphocytes	Lupus bodies	Normal nuclei	Hematoxylin bodies
Expressed as:	Amounts	Amounts	Amounts	Concentrations
No. measured:	20	20	30	15
Methyl green:	16.7±0.5	11.9±0.3	15.5±0.47	0.15±0.14
Methyl green after acetylation:	17.8±0.8	23.1±0.6	22.3±1.5	0.47±0.02
Feulgen:	19.9±0.4	21.3±0.5	17.8±0.5	0.43±0.02
$\frac{\text{Feulgen}}{\text{Methyl green}}$:	1.19	1.79	1.15	2.8
$\frac{\text{Postacetyl. Me. gr.}}{\text{Methyl green}}$:	1.06	1.94	1.43	3.26
$\frac{\text{Feulgen}}{\text{Postacetyl. Me. gr.}}$:	1.12	0.92	0.80	0.90

Successive measurements of: (1) methyl green binding capacity and the Feulgen reaction of DNA in free LE bodies derived from leukemic lymphocytes in an "atabrine" LE preparation, and comparable control nuclei of such lymphocytes; (2) the same hematoxylin bodies and normal renal tubular epithelial nuclei. Total amounts have not been calculated for the hematoxylin bodies because their irregular shapes make calculation of their volume difficult and inaccurate; only the extinctions (concentrations) are given. The ratios of both experiments may be compared, but the amounts in arbitrary units are comparable only within a given experiment (i.e., lymphocytes and LE bodies).

Note that acetylation of protein effects a greater rise of capacity to bind methyl green in nucleic acid of LE and hematoxylin bodies, and revises the Feulgen:methyl green ratios to the same general range as those of nuclei.

effects almost a twofold average rise in the ability of their DNA to bind methyl green. The ratio of the amount of methyl green bound after acetylation to that capable of being bound before acetylation gives us a measure of the DNA phosphate masked or pre-empted by combination with protein, and from these it would appear that about half of the stainable sites of DNA in lupus bodies were masked by protein, while less than one tenth of the nucleic acid phosphate was pre-empted in this way in the lymphocyte. Very similar ratios were obtained from measurement of LE preparations made by other methods.[43]

The results of such comparison of methyl green binding by normal renal tubular epithelial nuclei and hematoxylin bodies in kidney tissue fixed in Carnoy's fluid, from a case of systemic lupus erythematosus, are tabulated in Table I. The DNA of normal kidney epithelial nuclei exhibits a 43 per cent average increase in methyl green uptake following elimination of competing protein basic groups by acetylation. This larger rise, contrasted with only a 6 per cent increase in lymphocyte nuclei after this treatment, is a reflection of the smaller total amount of protein in the latter. In hematoxylin bodies in tissue, acetylation effects a more than threefold augmentation of the concentration of methyl green in the same bodies.

The methyl green binding capacity of hematoxylin bodies in tissues which had been fixed in formalin for long periods remained almost unchanged after subsequent acetylation, a result attributable[41,44] to the irreversible combination of formaldehyde with amino, imino and guanido groups. Formalin fixation thus resembles acetylation in its effect on methyl green binding.

Comparison of Methyl Green and Feulgen Staining

The Feulgen reaction for DNA is relatively insensitive to those changes in the state of DNA or in its relation to proteins that affect methyl green uptake. Measurements of the Feulgen reaction therefore provide a standard of reference with which other staining properties of DNA such as methyl green binding can be compared. For many types of nonproliferating cells, the ratio of the amount of methyl green-stained DNA to the amount of Feulgen-revealed DNA is nearly constant and happens to be around 1.0. In the data reported by Klemperer and co-workers,[7] the ratios of the Feulgen to methyl green reactions in hematoxylin bodies in tissue were found to be greatly enhanced as compared with normal nuclei, ranging from about 2.0 to 8.0, owing to depression of methyl green binding. These high ratios, which were at

that time interpreted as indicative of depolymerized DNA, may now be regarded as having been due, in some part, to protein interference. The methyl green binding of LE or hematoxylin bodies after acetylation of their protein, compared with the concentration of DNA measured in the same bodies by the Feulgen technique (i.e., the Feulgen: postacetylated methyl green ratio) may be expected to indicate whether there is any significant residual decrease in methyl green binding which cannot be accounted for by the presence of associated protein. The results, which are given in Table I, reveal that the Feulgen:postacetylated methyl green ratios of LE bodies and lymphocyte nuclei, and hematoxylin bodies and renal tubular epithelial nuclei alike are between 0.8 and 1.1 and thus tend to approach 0.1. It is therefore concluded that there is no significant decline of methyl green staining of DNA in lupus bodies which cannot be accounted for by protein interference, and that the DNA of the LE or hematoxylin body is not depolymerized or altered in state in a manner which is detectable cytochemically.

Comparison of the amounts of Feulgen-revealed DNA of lymphocyte nuclei and of whole nonphagocytosed LE bodies, each of which apparently originated from lymphocyte nuclei, make it clear that in the transformation of the leukocyte nucleus to the free LE body there is no appreciable loss in the total amount of DNA. After such bodies have been engulfed by polymorphonuclear leukocytes, evident loss of stainable material ultimately occurs. The reduction in the Feulgen stainability of hematoxylin bodies presumed to be of longer standing has previously been noted.[9,10]

PROTEIN

The foregoing data on nucleic acid stainability have drawn attention to the protein moiety of the nucleoprotein material which constitutes the hematoxylin or the LE body. The extent of its interference with methyl green uptake by the nucleic acid in these bodies suggests that their protein components differ both in kind and amount from those normally present in nuclei.

Proteins of the Normal Nucleus

From about 75 per cent (in lymphocytes) to 95 per cent (in liver cells) of the dry mass of normal nuclei isolated in nonaqueous media is made up of protein.[45,46] In contrast to the constancy of the DNA complement of each nucleus, the dry mass may vary considerably in amount and in kind of different cell types of the same organism, and in the same cell types in different physiologic and pathologic states.[47-54]

Closely associated with DNA in all somatic nuclei are the strongly basic histones, which presumably exist in the nucleus as salt-linked histone deoxyribonucleates,[47,54,55] and are present in definite quantitative ratio to DNA in any cell type.[20,47,55] They are less active metabolically than other nuclear proteins, as judged by isotopic turnover rates.[47] Also associated with DNA as components of the somatic chromosome, are higher proteins which have been designated "residual chromosomal protein."[53] The amounts of "residual" proteins depend upon cell type and metabolic activity.[47,52,53] Liver and kidney cell nuclei, for example, have more such protein than lymphocyte nuclei.[47,52] Protein of the "residual" type is particularly effective in interfering with methyl green binding to the DNA with which it is associated, and this fact is reflected in the higher Feulgen:methyl green, and postacetylated methyl green:methyl green ratios of kidney nuclei as compared with those of lymphocyte nuclei (Table I). In addition to those named, the normal nucleus also contains other, hitherto little-studied proteins, presumably not chromosomal, among which there is a soluble globulin and a lipoprotein.

Proteins of the LE Body

"Total" Protein. Quantitative measurement of the changes in amount of protein resulting from the LE transformation necessitates comparison of the protein content of LE bodies, each of which is known to be derived from a whole nucleus, with that of the type of nuclei from which they originate. Such cytochemical determinations were made on lymphocyte nuclei and free LE bodies resulting from transformation of lymphocyte nuclei in atabrine preparations.[18]

Stoichiometric binding of flavianic acid (naphthol yellow S, the dipotassium salt of 2,4-dinitro-1-naphthol-7-sulfonic acid) to *available* basic (ε-amino, guanido and imidazole) groups of the dibasic protein residues provides the basis for their quantitative determination, and hence an index of the total amount of protein.[22] The concentration of Feulgen-revealed DNA can be measured in the same object concurrently with that of naphthol yellow S, due to the wide separation of the respective absorption peaks (435 mμ and 568 mμ) of the colored complexes.[22] This was done in these determinations in order to visualize and delimit the objects, since visual definition with naphthol yellow S alone proved insufficiently accurate. Since nucleic acid was not extracted prior to staining, the relative amounts of naphthol yellow S measured in these experiments represent only those protein basic groups not pre-empted by combination with DNA, rather than the

potential total demonstrable number. Microspectrophotometric measurements of such material show that more than a twofold increase in naphthol yellow binding capacity occurs in the LE transformation from lymphocyte nuclei, and there is a change in the naphthol yellow S:Feulgen ratio of from 0.82 for lymphocytes to 1.93 for LE bodies.[56]

These values point to an increase in protein basic groups and suggest that the LE change involves an actual increase in the amount of protein. The results of the cytochemical Millon reaction for tyrosine residues of the protein further indicate that this is the case. There are more than twice as many Millon-reactive tyrosine residues in the LE bodies than in the parent lymphocyte nuclei.[56] Interferometric measurements have also revealed that a more than twofold gain of dry mass is entailed in the transformation of nuclei to LE bodies.[57] From these data it is concluded that an actual augmentation in the total amount of protein occurs in the LE transformation of nuclei.

Histones. Owing to their high isoelectric point, strongly basic proteins like histones (which are rich in arginine and lysine residues) can be selectively and quantitatively stained ***in situ,*** after removal of nucleic acids, by the anionic dye fast green FCF at alkaline pH.[20,21] A modification of the Feulgen procedure, in which the molar substitution of trichloroacetic acid for hydrochloric acid insures retention of protein during hydrolysis and staining, permits the successive demonstration of DNA and then histone in the same body.[20] Utilizing this method, it has been shown that histone is present in all normal and pyknotic chromatin, and has precisely the same microscopic distribution as DNA.[20,21] Some results of the application of these techniques to the hematoxylin bodies of the tissues are shown in Figures 4 and 9. Neither in these nor in the inclusions of typical LE cells (Figs. 14 and 16) can stainable histones be detected. The free unengulfed LE bodies in LE preparations of different kinds are diffusely and relatively faintly stained (Fig. 15), or sometimes quite unstained for histones. Some of the bodies early in the course of transformation show irregular diffuse staining of some parts, and absence of staining in others. Microspectrophotometric measurements of the histone in bodies very early in the course of their evolution from lymphocytes confirm the visual impression of a striking decrease of stainable histone in them, while the concentration of stainable histone in fully formed LE bodies is below accurately measurable limits.[56] The LE change involves a dissolution of chromatin structure and either a loss of histones or some change which renders them vulnerable to preparative loss, or else a masking of their stainable groups. The Sakaguchi reaction for arginine

residues is not affected by those electrostatic factors which influence acid and basic dye binding, and affords a useful check on the fast green technique. Thus, the marked diminution of arginine stainability of LE and hematoxylin bodies as compared with nuclei suggests that an actual loss of histones occurs in the formation of LE or hematoxylin bodies. In contrast to the strong PAS stainability of hematoxylin bodies in tissue, the LE bodies fail to color after the PAS reaction; neutrophil cytoplasm is well stained.

Discussion

The cytochemical method of analysis affords a practicable and feasible approach to an examination of the composition of hematoxylin and LE bodies, in spite of the relative paucity of chemically specific colorimetric reactions available. Through knowledge of the composition of these bodies, and of the ways in which this differs from that of the nuclei from which they take origin, it was hoped to gain some insight into a pathogenetic process operative in systemic lupus erythematosus.

Previous cytochemical interpretations[7-9] of the lupus bodies did not take into account those factors, other than the state of polymerization of DNA, that influence methyl green binding to nucleic acid. Indeed, the effects of fixation,[31,51,59] molecular size,[59] pH and ion competition,[39,51] and the presence of protein[30,40,51,59] on the uptake of basic dyes by nucleic acid have only recently been given sufficient attention, especially as they relate to methyl green staining.

Knowledge of these variables provides us with a means of controlling some of them, and in this way probing into the state of the nucleoprotein complex and the relationship of DNA to protein in various cell states.[51,52] The data presently reported indicate that the impairment of methyl green binding to nucleic acid in both hematoxylin and LE bodies is due to interference by competing protein which is not present in nuclei from which such bodies may form. The restoration of the Feulgen:methyl green ratio of LE and hematoxylin bodies, after destruction of the basic groups of competing protein, to values approaching those of nuclei would indicate that there is no cytochemically detectable change in the state of polymerization or molecular configuration of the DNA in these bodies. Moreover, there is no loss of Feulgen-demonstrable DNA in the original LE transformation.

Inquiry into the quantity and kinds of protein associated with the nucleic acid in the LE body confirms the suggestion of the data provided by methyl green binding that the conversion of nuclei to LE

bodies entails a marked increase in protein and hence in competing protein basic groups. Nonhistone protein, such as chromosomal "residual protein," has been found to be more effective than histone in depressing methyl green stainability of DNA in interphase nuclei.[51] This fact indicates that the masking action of protein with respect to methyl green stainability of nucleic acid phosphate groups is not solely a property of the number of positively charged groups on the protein. Similarly, in the LE body, despite the apparent loss of histone, an unusual protein of higher type becomes linked to DNA and masks more than half of its methyl green stainable anionic sites.

In the formation of the LE body, protein normally foreign to the nucleus enters into it. This would appear to be an initial event.[57] The resulting nucleoprotein mass differs markedly in composition from that in the original nucleus. The data permit one to hypothesize that this protein, at least in part, effects a displacement of histones from their usual linkage with DNA. This would result in their susceptibility to degradation, loss or recombination and masking. The foreign protein itself becomes associated with DNA in such a manner as to interfere with methyl green staining. There is no evidence from the cytochemical data to support the view that the LE phenomenon primarily affects the DNA molecule.

Previous investigations[10] of the protein of the hematoxylin bodies were concerned with their contribution to the formation of fibrinoid material and hyaline thrombi. The hematoxylin bodies and the material remaining after hot trichloroacetic acid extraction were shown to give positive reactions with a modification of the Millon reaction developed for cytochemical use.[23] It was concluded, therefore, that these substances contained tyrosine residues. They were also found to be readily digested by trypsin at pH 7.3. On the basis of their staining with the aniline blue-orange G mixture at pH 3.0, it was inferred that the bodies also contained a basic protein,[10] at first believed to be histone. According to White,[60] histone and globin in purified state and in tissues from which the nucleic acids had been extracted by trichloroacetic acid, bind orange G selectively from the dye mixture. However, any protein possessing basic residues may bind anionic dyes such as orange G at low hydrogen ion concentration. Specific characterization of a protein which stains with orange G under these conditions would now seem to be of questionable validity.

Gueft and Laufer[10] stated that there was no difference in optical density of the Millon stained hematoxylin bodies before and after use of the mercuric sulfate-sulfuric acid reagent in the technique developed

by Pollister and Ris[23] for detection of total and nonhistone protein. However, no detailed data on this point were published by Gueft and Laufer.[10] The observations with the Millon reaction, and those presently reported indicating failure of hematoxylin bodies to bind fast green at pH 8.1, would seem to show that histone is absent from this protein moiety. The other characteristics of the protein residue, such as insolubility in hot trichloroacetic acid or the sulfuric acid Millon reagent, its digestibility by trypsin, and its relative acidophilia, are shared by most nonhistone, nonprotamine proteins, including the "residual chromosomal protein" of Mirsky and Ris.[58] These, however, cannot be considered as specific or even characteristic qualities of any class or group of proteins.

The view of Gueft and Laufer[10] that the "protein constitution of the hematoxylin body does not differ from that of the normal nucleus" is surprising, and cannot be accepted on the basis of present evidence. Indeed, in the failure to demonstrate stainable histone alone, the protein of the hematoxylin body differs profoundly from that of all normal and pathologic nuclei thus far studied.

Further indications of the origin and nature of the incurrent protein found in the LE body have recently been provided by the use of fluorescent labeled antibodies for histologic localization. Mellors and co-workers,[61] and Vazquez and Dixon[62] have localized human gamma-globulin by this means in the inclusions of LE cells, and have noted its absence from normal nuclei. Friou, Finch and Detre[63] have observed that fluorescent antibodies to globulin derived from patients with systemic lupus, but not others, become localized in nuclei when applied to normal mouse tissues. It is of interest that gamma globulin has been localized in the fibrinoid alterations of arterioles and glomeruli in lupus.[64] Moore, Weisberger and Bowerfind[65] have claimed that the protein and carbohydrate material of hematoxylin bodies in lymph nodes are derived from intracytoplasmic bodies which develop in plasma cells in the nodes.

The nature of the reaction of the protein with the nuclear constituents remains unknown. Any explanation of the LE phenomenon must take into account the fact that substrate cells must have been previously traumatized. It is also necessary to take into consideration the rapidity with which the phenomenon occurs[57] and its lack of specificity with respect to species and cell type.

The chemical constitution of the free LE body cannot be regarded as identical with that of the LE cell inclusion, which may have undergone intracellular digestion by the polymorphonuclear leukocyte. For this

reason, in order to gain some insight into the primary pathogenetic effects of the lupus factor on cell nuclei, the free LE body has been used for most of the examinations in this study.

While there can be little doubt that the hematoxylin body is derived from the LE body, histochemical studies make it obvious that substances added to and dissipated from the hematoxylin body in the course of its sojourn in the tissues may alter its composition secondarily. For example, LE bodies produced *in vitro* have undiminished content of DNA; they are metachromatic[48] and fail to react after the PAS procedure. Moreover, older hematoxylin bodies in tissue apparently often have reduced Feulgen stainability; they are not metachromatic, and they react strongly with the PAS test.[10,65] The original nucleoprotein material (LE body) resulting from the action of the lupus factor on susceptible nuclei thus undergoes further changes in the tissue. These appear to be characterized by aggregation of the bodies, the addition of extracellular protein and of PAS-reactive carbohydrate.[10,65] There is also gradual loss of DNA[10] and possibly of other materials. Gueft and Laufer,[10] and Klemperer[9] believe that such hematoxylin bodies may appear as extracellular proteinaceous deposits from which nucleic acids have disappeared. These may then constitute the fibrinoid material of systemic lupus erythematosus.

Summary

LE bodies formed *in vitro*, and hematoxylin bodies from the tissues of 3 cases of systemic lupus erythematosus were examined cytochemically. Binding of methyl green by the DNA of these bodies was compared with control nuclei and found to be depressed, with consequent elevation of their Feulgen:methyl green ratios. Destruction of competing basic groups of the protein associated with DNA in these bodies increased methyl green uptake by 2 or 3 times, and restored the Feulgen:methyl green ratios to values similar to those of control nuclei. The amount of DNA was found to be undiminished in the conversion of a lymphocyte nucleus to an LE body. These data would indicate that the DNA of LE and hematoxylin bodies was not demonstrably depolymerized, and that a protein of a kind or amount not normally present in nuclei was linked to the DNA of these bodies.

The formation of LE bodies was found to entail large increases of protein demonstrable by means of naphthol yellow S (flavianic acid) binding and the Millon reaction. At the same time histones, normally linked to DNA in nuclei were found to be apparently diminished or lost. It is postulated that in the LE transformation there was an

influx of protein normally foreign to nuclei, a displacement of histone from its combination with DNA, and an association of the DNA with the new protein.

The nucleoprotein mass formed in such a pathogenetic process appeared to undergo further changes in the tissues, giving rise to more mature hematoxylin bodies.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Skin biopsy, showing hyaline thrombi and hematoxylin bodies, indicated by arrows, in dilated capillary vessels of the upper corium. The hematoxylin stained smudges in some of the hyaline material are noteworthy. Hematoxylin and eosin stain. × 300.

FIG. 2. Identical field stained with methyl green for DNA, illustrating methyl green binding capacity of hematoxylin bodies. Methyl green stain. × 300. Red filter.

FIG. 3. Identical field after application of TCA-Feulgen procedure. The same bodies are stained by the Feulgen reaction. × 300. Yellow-green filter.

FIG. 4. Identical field after the alkaline fast green technique for histone. The hematoxylin bodies and smudges fail to stain; their locations are marked by arrows. × 300. Red filter.

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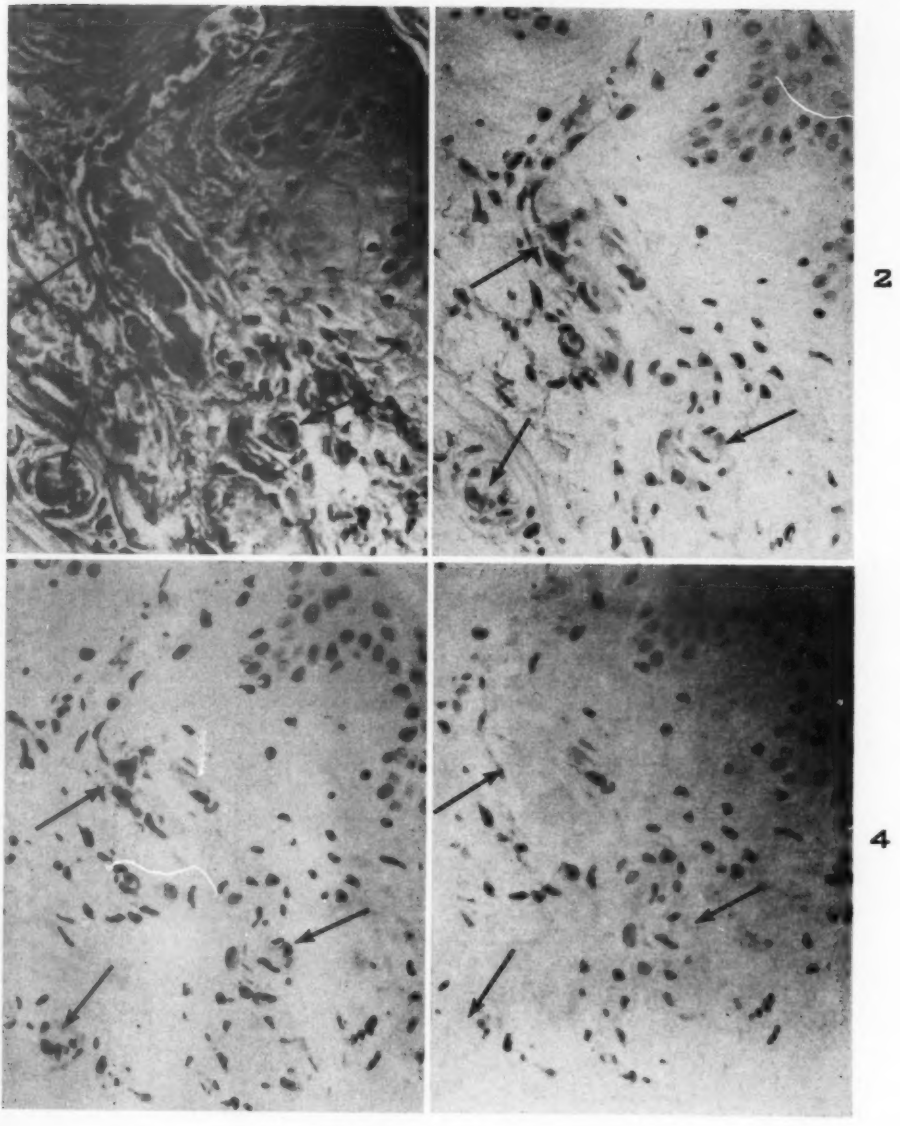
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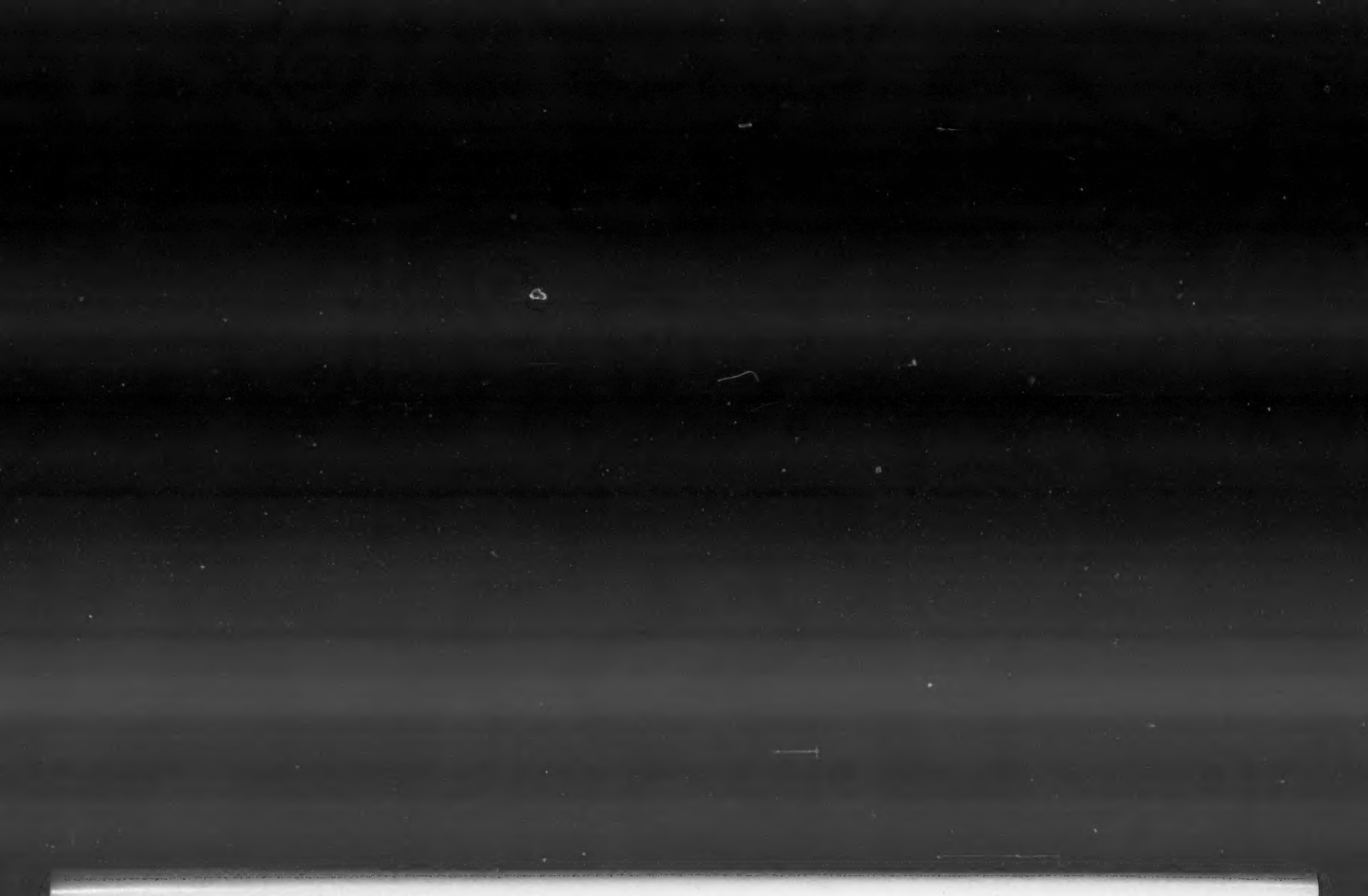
FIG. 5. Skin biopsy. Hyaline thrombi and hematoxylin bodies, the latter indicated by arrow, and smudges in vessels of the corium. Hematoxylin and eosin stain. × 300.

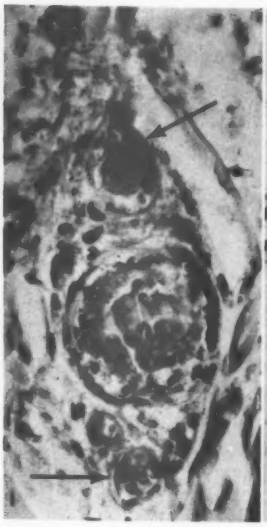
FIG. 6. Identical field, stained for DNA with methyl green stain. × 300. Red filter.

FIG. 7. Identical field, restained with methyl green after acetylation. Some increase in the intensity of staining of the hematoxylin bodies is evident after the elimination of potentially competitive protein basic groups. Methyl green stain. × 300. Red filter.

FIG. 8. Lymph node. Large aggregate or packet hematoxylin body in medullary channel of lymph node in which there is extensive necrosis. TCA-Feulgen procedure. × 450. Yellow-green filter.

FIG. 9. Same hematoxylin body after alkaline fast green technique for histone, showing failure to stain. The sharp outlines of the body in some areas are caused by difference of refractive index between tissue and medium. Alkaline fast green stain. × 450. Red filter.





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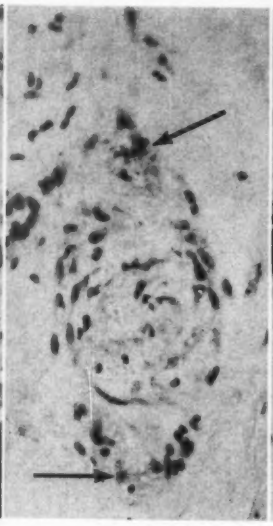

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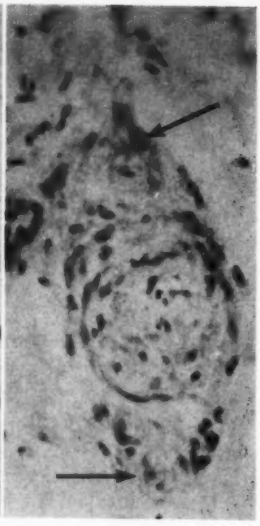

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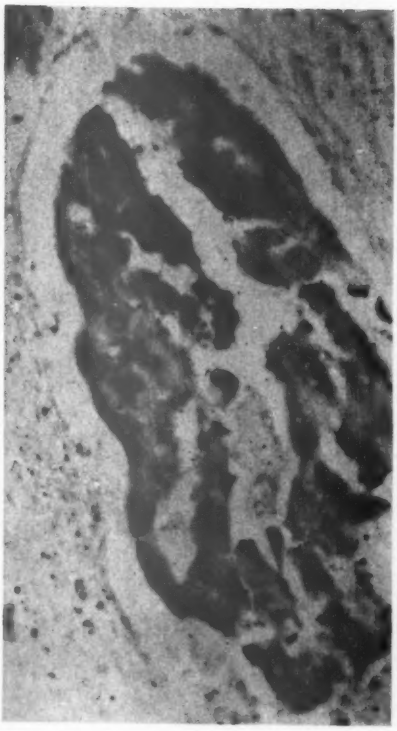

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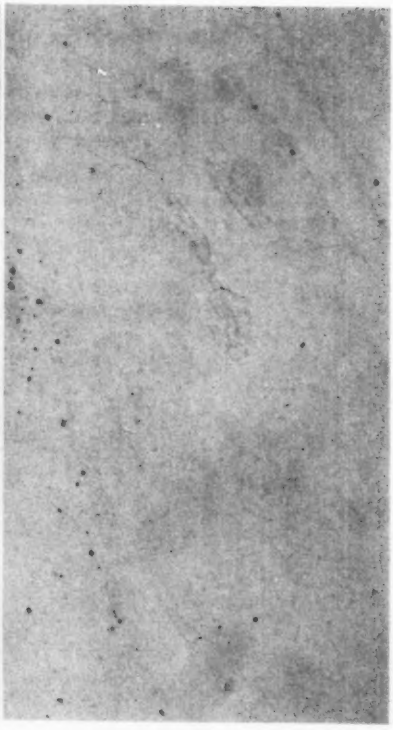

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FIG. 10. LE preparation from normal human buffy coat made by the dried substrate method.[17] All of the LE bodies shown in the field have been engulfed by phagocytes. Wright's stain. × 900.

FIG. 11. LE preparation from buffy coat of a patient with lymphocytic leukemia, made by the atabrine method.[18] The converted bodies are enlarged and pale. Such bodies are susceptible to phagocytosis on the addition of viable polymorphonuclear leukocytes. Wright's stain. × 900.

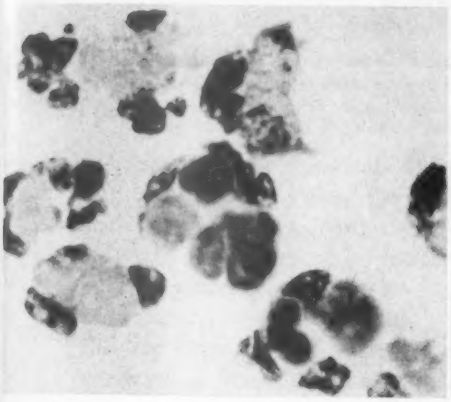
FIG. 12. Free LE bodies (arrow) and LE cell inclusion, in a dried-substrate preparation, stained with methyl green for DNA. × 920. Red filter.

FIG. 13. Same field as that shown in Figure 12, after acetylation and restaining with methyl green. Intensification of staining is evident, especially in the LE bodies. × 920. Red filter.

FIG. 14. LE cells and polymorphonuclear leukocytes in a dried substrate preparation, stained with alkaline fast green method for histones. The nuclear chromatin stains well; the LE inclusions (arrows) are unstained. × 920. Red filter.

FIG. 15. A relatively intact polymorphonuclear leukocyte (upper) and a leukocyte (lower) the nuclear lobes of which have undergone the early changes of the LE transformation. The fast green stainability of the latter is considerably diminished. Alkaline fast green method. × 920. Red filter.

FIG. 16. Polymorphonuclear neutrophils, an eosinophil and two LE cells in a dried substrate preparation, stained with the alkaline fast green method for histones. The chromatin of the nuclei and basic protein of the eosinophil granules are colored; the LE inclusions fail to stain. × 920. Red filter.



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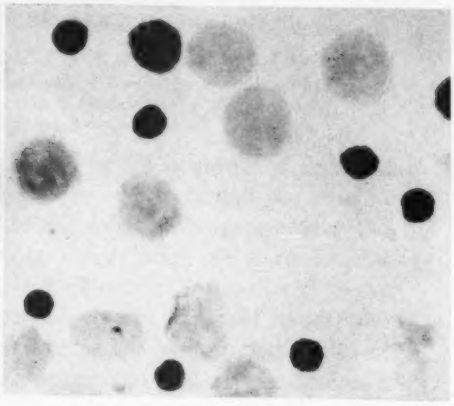

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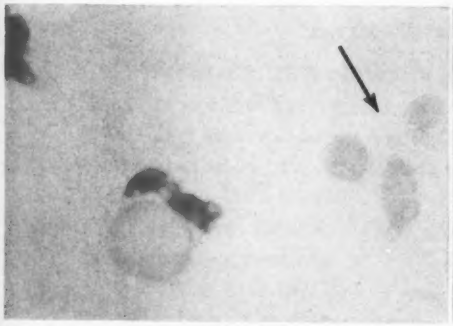

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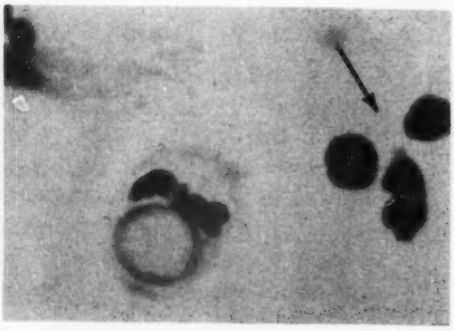

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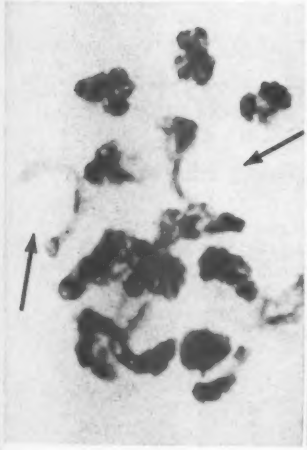

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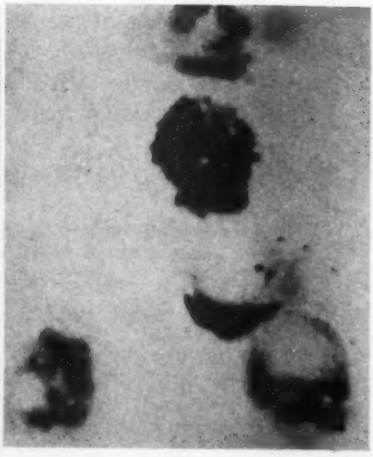

16

RACHITOMIMETIC EFFECTS OF FLUORIDE FEEDING ON THE SKELETAL TISSUES OF GROWING PIGS*

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In spite of the numerous approaches to the problem of fluorosis during the past 30 years,[1] the pathogenesis of the changes observed in skeletal tissues remains unclear. While some authors[2] consider the lesion to be a form of osteosclerosis, others attribute it to mineral deficiency characterized by an increase of osteoid formation.[3-5] Some consider the osseous condition a response to parathyroid hyperfunction[5] or intoxication;[6] others have reported the aggravating effects of a calcium deficient diet.[7] Studying young dogs, Kellner[8] recognized a gross similarity between the bony changes in fluorosis and rickets. Our present survey of growing pigs casts some light on this problem.

Materials and Techniques

In this study, tissues from 12 castrate male Hampshire pigs,† 6 weeks of age and averaging 65 lbs. in weight were used.[9] The animals, which had been raised on a normal farm ration, were paired, and one member of each pair was placed on a diet containing 1,000 parts per million of sodium fluoride. Thereafter the animals were pair-fed so as to insure equal intakes except, of course, for fluoride. After 30 days of feeding, each animal received an intravenous injection of 6 mc. of $S^{35}O_4$. Pairs were sacrificed at 10 minutes, 5 hours, 24 hours, 10 days, 30 days, 60 and 90 days.

Ribs and the heads of metatarsals were fixed in formaldehyde, demineralized in nitric acid and sectioned in paraffin. These sections were stained with hematoxylin and eosin, hematoxylin-phloxine B and orange G (HPO), Masson trichrome,[10] toluidine blue, the periodic acid-Schiff stain,[11] and the von Kossa stain. Some sections were examined unstained with phase contrast microscopy. Other sections were incinerated by the technique of Scott.[12]

A parallel histologic and histochemical study was made on the

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† Tissue samples were made available from animals raised and treated at the UT-AEC Agricultural Research Program.

teeth.[13] Autoradiographic observations were carried out *in vivo*[14] and *in vitro*[15] in order to determine the uptake of radiosulfate and radiocalcium.

Observations

Distortion of the linear pattern of the epiphyseal plate chondrocytes, encroachment upon the hypertrophic zone by bone forming tissue, decrease in the size of the spicules and increased osteoid production have been reported in previous similar experiments.[16] In the present series, the bones were studied only after demineralization and embedding in paraffin. A marked difference was observed between fluorinated and control animals in respect to the diameter of the costochondral junction of the ribs. While the outer diameter of the diaphysis was comparable in both cases, the heads of the bones of fluorinated animals had a "beaded" appearance (Fig. 1) and were approximately twice the size of the controls at 90 days.

In the newly deposited osseous material, striking histologic differences were also observed. Osteoid, otherwise called prebone,[17] stained orange with the HPO stain and green with the Masson technique. The central portions of the trabeculae showed red acidophilic material which increased rapidly in thickness, so that in the more differentiated tissue (secondary spongiosa), only a narrow border of yellow or green prebone was visible.

The alveolar bone of fluorinated animals showed progressive changes comparable to those of dentine and cementum reported elsewhere[13] with gradual decrease of acidophilia and the formation of bright globular bodies. These appeared in the more mature trabeculae and were rather uniformly distributed. Subsequent changes consisted of irregular growth and enlargement of the trabeculae. The marrow cavities also became enlarged and contained distended blood vessels. The modified bone matrix as well as the cementum of fluorinated animals frequently had a fibrillar appearance, indicative of a decrease in the binding substance.

In the fluoride-treated animals at 60 and 90 days, there was evidence of increase in the number of osteoblasts in productive areas. On the other hand, osteoclasts were also more numerous. Some of them contained one or several bright intracytoplasmic globular masses.

Periosteal bone appeared to be affected more slowly. At the epiphyseal plate, the spicules did not show any globular material but appeared to grow progressively thinner and more fragile, becoming easily detached from the cartilaginous plate (Fig. 1).

The progressive hypertrophy of the cartilage head of long bones was

most impressive (Fig. 1). The stained sections indicated that this condition was due to an increase in the mitotic rate of cartilage cells and a state of immaturity associated with decrease in the formation of matrix. The outcome was a highly cellular pale-staining cartilage, invaded by blood vessels and apparently soft and fragile. The epiphyseal plate showed patchy degeneration with invasion by vascular bone-forming tissue from the shaft marrow (Fig. 2). The bone spicules grew over progressively thinner cores of cartilaginous tissue. There were no globular masses in cartilage at any time.

The cartilage of the head of long bones has been reported to show a decrease of metachromasia in the zone of mineralization.[18] In the present series, there was no apparent decrease in staining intensity in the epiphyseal plate of fluorinated animals. However, the color produced by toluidine blue at this level appeared to be violet-blue rather than magenta as in normal cartilage. When stained with toluidine blue, the sections of normal bone, particularly the newly formed trabeculae, were weakly metachromatic. The reaction was most apparent in the vicinity of the osteocytes. By comparison, the bone of the fluorinated animals did not stain metachromatically but exhibited a pale blue quality.

With the PAS stain[11] prebone appeared to be practically unstained. Normal bone showed a gradation from moderate to strong staining toward the center of the trabeculae. The bones of the fluorinated animals showed, as in the case of the dentine and cementum matrix,[13] a progressive decrease of PAS staining intensity.

With phase contrast microscopy, the normal cartilage matrix appeared dark under the conditions of observation. In fluorinated animals, fine bright granular material appeared in the matrix between the rows of hypertrophic cells (Fig. 3). These small bodies were increased in quantity, but not in size, near the region of bone growth. They were also more abundant in animals fed the fluoride diet for 60 and 90 days. Large, bright globular masses, comparable to those previously recognized in dentine and cementum,[13] were disseminated throughout the newly formed bone trabeculae (Fig. 4). These also appeared in the marrow spaces in the vicinity of growing bone.

All the tissues were received in demineralized state and failed to react to the von Kossa stain for lime salts after a maximal exposure of 60 minutes to a 1.5 per cent solution of silver nitrate.[19] The incinerated preparations of demineralized tissues were not expected, of course, to yield much ash. However, a recent series of experiments[20] revealed an intercellular distribution of blue-white ash in cartilage and other

soft tissues such as epidermis, hair, retina, etc., corresponding to regional Ca^{45} uptake.

The incinerated sections of demineralized normal pig cartilage contained a small proportion of intercellular blue-white dust and also relatively abundant localized residues of white ash reproducing the shape of cartilage cells. The demineralized sections of cartilage from the fluorinated animals yielded an increased amount of ash, comparable in appearance to normal cartilage ash and located between the rows of hypertrophic cells (Fig. 5). The newly formed bone trabeculae also produced a modified spodogram in which ash appeared more abundant in the outer portion of the trabeculae when compared to animals on the fluoride diet for 30 days (Fig. 7). At 60 days, the ash was more abundant and more uniformly distributed in the trabeculae which had developed during the period of fluoride feeding (Fig. 8).

For purposes of comparison, sections of the costochondral junctions of the ribs of 3 human children suffering from vitamin D deficiency rickets were also incinerated. The spodograms revealed an increased ash content, particularly abundant in the vascularized zone, characteristic of this disease (Fig. 6).[21]

It is possible to consider the ash content of the fluorinated bones to represent original deposits of calcium fluoride. This has been reported previously as a probable occurrence in fluorosis.[22] Calcium fluoride is known to be only slightly soluble in acids used in the usual techniques of demineralization. On the other hand, this substance is soluble in solutions of ammonium salts.[23]

Sections of bones of fluorinated animals were placed in a 2 per cent aqueous solution of ammonium acetate for 24 hours. They were then incinerated or mounted unstained for study by phase contrast microscopy. After this treatment, the majority of the globular masses were found to have disappeared from the bone trabeculae; some were actually observed to be partially dissolved. On the other hand, the substance which yielded the diffuse, fine ash did not seem to be affected by the ammonium acetate.

Discussion

Some of the manifestations of fluoride feeding reported here and in previous work[16] recall the classical rachitic syndrome: decreased growth, decreased and imperfect mineralization, hypertrophy of the costochondral junction, and overproduction of osteoid (Fig. 1). However, these may in part represent reactive, secondary changes. While the previously laid out portions of the skeleton appeared to be barely

involved, bone formed during the period of fluoride feeding was greatly modified in both its mineral and organic content.[16,24] Indeed, the epiphyseal cartilage increased in size and became softer so that weight bearing produced a distortion of the architecture.[16] On the other hand, autoradiographic records of tracer doses of $S^{35}O_4$ revealed that the tagged sulfate disappeared from fluorinated cartilage at a slower rate[14] and did not diffuse readily from predentine into dentine. The polysaccharide content of bone as well as that of dentine and cementum seemed to have decreased as evidenced by the toluidine blue and PAS reactions. However, in the fluorinated animals, the new trabeculae were larger and irregular.

The hypertrophy of the cartilage, bone and dentine is not conducive to growth of the animal and seems to be the result of accumulation and overproduction of an abnormal matrix. In bone and dentine, this substance appears immature, with staining affinities comparable to those of prebone and predentine. On the other hand, the bone and cartilage matrix of the fluorinated pigs contains some material which resists acid demineralization and yet may be a salt since it yields abundant ash in the process of micro-incineration. Deposition of CaF_2 in bone under similar conditions has long been suspected[2,8,22] and has recently been established by F^{18} autoradiography.[25]

The present experiments have revealed that only portions of the structural mass, presumably those containing salts, were dissolved in ammonium acetate, a solvent of CaF_2. These were the large globular masses observed in bone, dentine, cementum and some peripheral connective tissue structures, such as tendons and ligaments. They are, presumably, the *Kalkhörner* of Kellner,[8] and are considered responsible for the sclerosing character of the disease.[2]

In the areas of normal mineralization or premineralization, a large amount of blue-white ash, resistant to acid demineralization and to ammonium acetate, appeared in the spodograms. In cartilage, this pattern was comparable to that of vitamin D deficiency rickets in children (Fig. 6) and to unpublished observations on strontium rickets in the rat. As in these two hypertrophic disorders, here also there was an intense uptake of Ca^{45} *in vitro*.[15,20] It is thus possible to consider that this diffusely distributed material may represent not CaF_2 but an organic salt of calcium. The existence of an organic precursor to mineralization has been postulated by Sobel (the intrinsic factor).[27] On the other hand, Newman, Boyd, and Feldman[28] have shown that chondroitin sulfate can behave as a cation exchanger *in vitro*. It is possible that the blue-white ash deposits in fluoride intoxication may

represent an accumulation of chondroitin holding calcium as a loose combination or as a more stable, unnatural salt. Thus, calcium would be deposited in sites of growth, partly as CaF_2, partly as an organic salt, accumulating since normal mineralization cannot occur. The latter condition apparently also prevails in vitamin D deficiency and in strontium rickets.

Summary

Young pigs, fed for 30, 60 and 90 days on a diet containing 1,000 parts per million of sodium fluoride, have shown defective growth and mineralization of bones, costochondral beading, softened and deformed epiphyseal plates, and enlarged and malformed bone trabeculae.

Histochemical studies of demineralized sections have revealed a decrease in the stainable polysaccharides and an accumulation of salt, the solubility of which resembled that of calcium fluoride. The larger portion of the deposit observed in spodograms seemed related to an organic calcium combination, the significance of which is discussed in relation to the mechanism of mineralization and is compared with vitamin D deficiency and strontium rickets.

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[*Illustrations follow*]

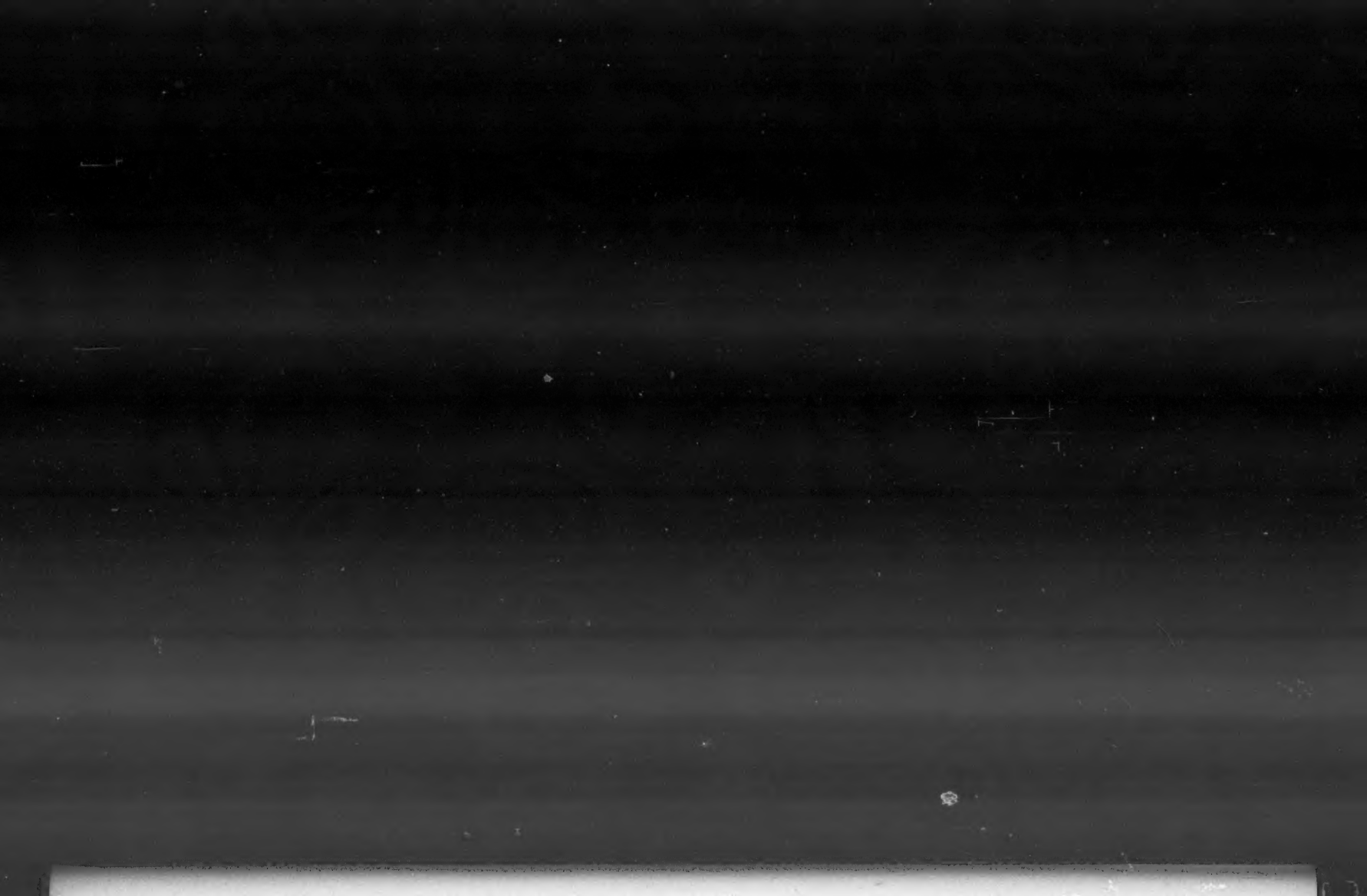
LEGENDS FOR FIGURES

FIG. 1. The costochondral junction of the rib of a pig 20 weeks of age on fluoride diet for 90 days. The diameter of the rib at the epiphyseal plate is approximately twice that of the shaft. In the control animal, these measurements are roughly identical. Hematoxylin-phloxine B and orange G (HPO) stain. × 3.

FIG. 2. The border of an epiphyseal plate modified by fluoride diet. Note the localized zone of cartilage destruction and, in the upper portion of the picture, deep penetration by marrow constituents. HPO. × 57.

FIG. 3. The epiphyseal cartilage of bone from a pig on fluoride diet for 60 days. In this demineralized section the bright dust in the matrix and the bright cytoplasm of the chondrocytes may be noted. Dark M phase contrast microscopy. × 200.

FIG. 4. A trabecula of alveolar bone from a pig on fluoride diet for 30 days. Note the bright globular masses in the bone and in lesser amount in the marrow spaces. There is no brilliant dust visible. Dark M phase contrast microscopy. × 200.



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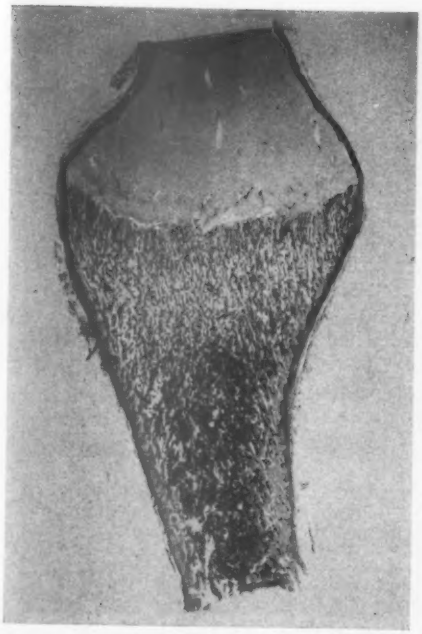

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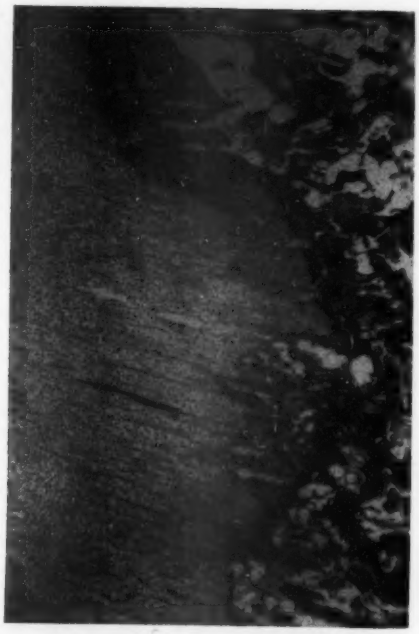

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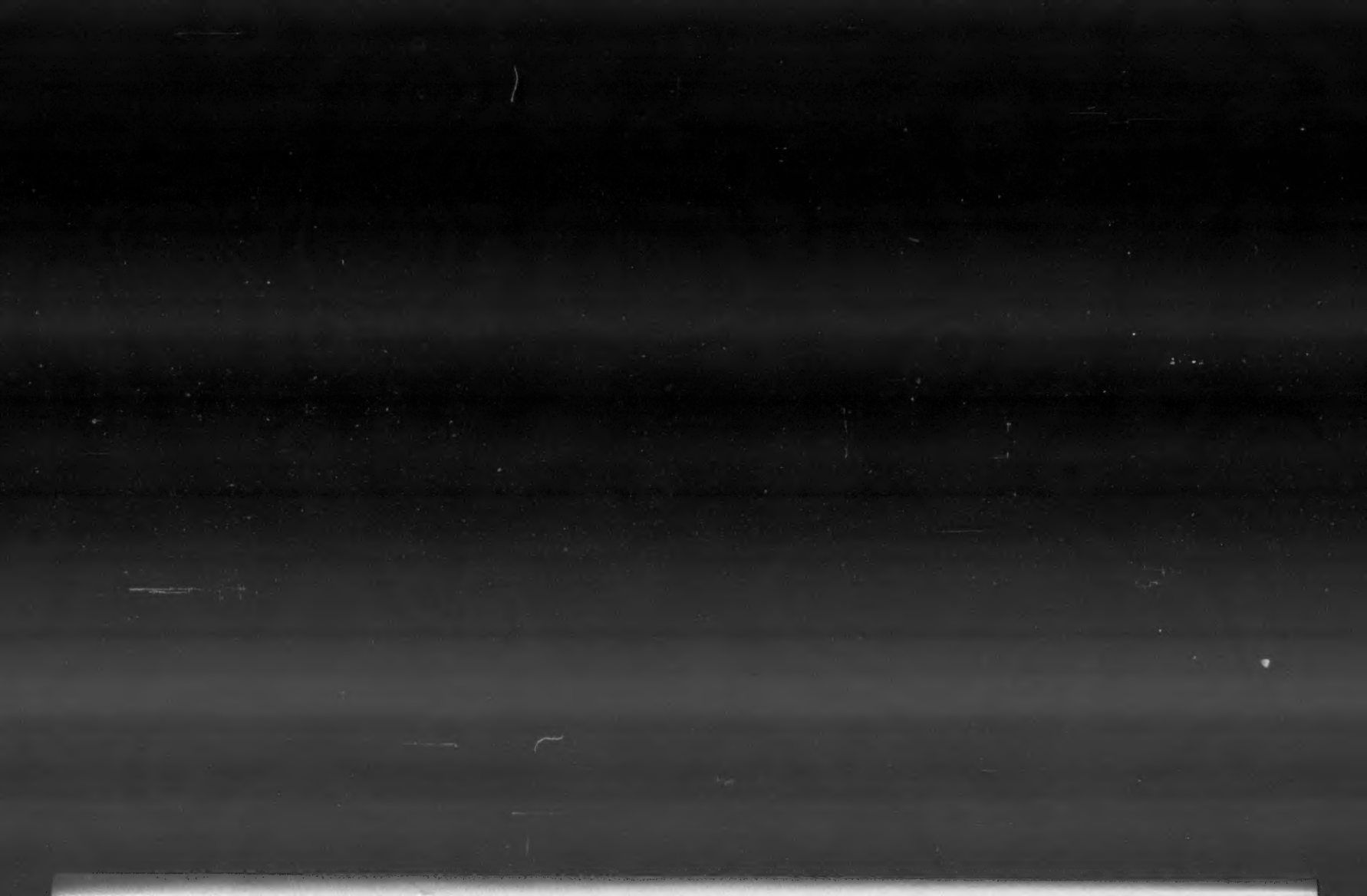
FIG. 5. Spodogram of epiphyseal cartilage of a pig on fluoride diet for 60 days. There is increase of matrix ash near the shaft margin of the plate. Phase contrast microscopy. × 100.

FIG. 6. Spodogram of a demineralized section of vascularized portion of rib at the costochondral junction. A 3-year-old child with vitamin D deficiency rickets. A heavy ash deposit is apparent in the cartilage matrix. Phase contrast microscopy. × 100.

FIG. 7. Patchy distribution of fine particles of blue-white ash in a newly formed bone trabecula of a pig on fluoride diet for 30 days. Spodogram. × 200.

FIG. 8. Homogeneous distribution of fine particles of blue-white ash in a bone trabecula of a pig. This has developed during 60 days of fluoride feeding. Note also the denser, ovoid masses corresponding to the globular bodies in Fig. 4. Spodogram. × 200.



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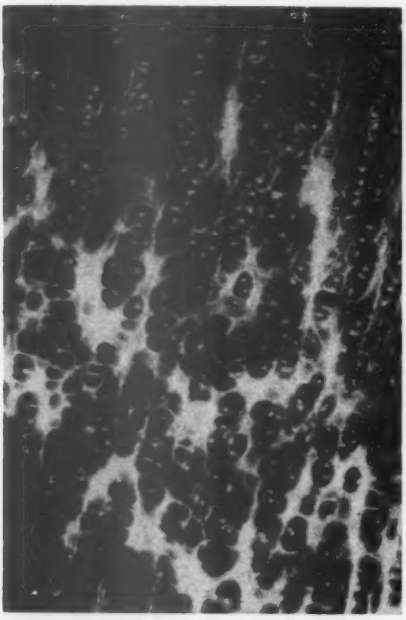

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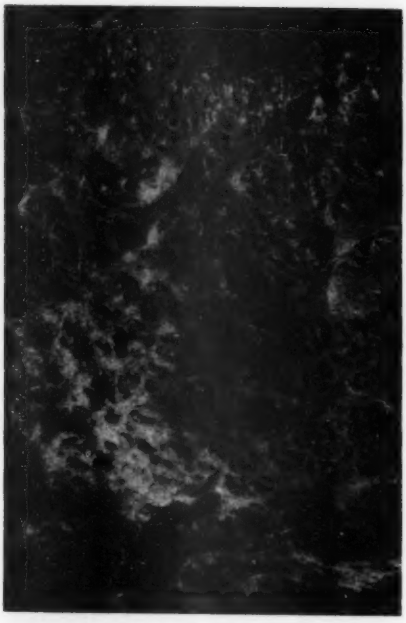

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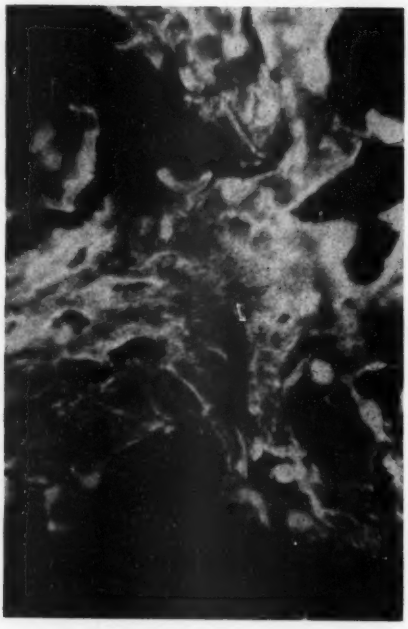

8

HYPERVITAMINOSIS D IN MONKEYS;

A Clinical and Pathologic Study *

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The Radiobiological Laboratory of the University of Texas and the United States Air Force, Austin, Texas, and the School of Aviation Medicine, Randolph Air Force Base, Texas, maintain a monkey colony in connection with an experimental program in radiobiology. Because of an error by the manufacturer of the monkey food, the entire colony received excessive amounts of calcium, phosphorus, and vitamin D for a period of nearly three months. Acute vitamin D intoxication developed and resulted in a number of deaths before the diagnosis was established. This opportunity of observing vitamin D intoxication in a large number of higher mammals was not a planned experiment, but developed accidentally. The clinical and pathologic observations made while the colony received the toxic diet and for one year thereafter are the subject of this report.

Clinical Information

At the onset of the high vitamin D diet, there were 558 monkeys (*Macaca mulatta*) in the colony: 283 were in the Austin colony and 275 were in a holding colony at Randolph Air Force Base. Of the 283 animals in the Austin colony, 103 had received whole-body ionizing radiation; 70 had received focal ionizing radiation to the right eye; 21 had received intravenous nitrogen mustard; 89 were control animals which had received neither ionizing radiation nor nitrogen mustard. Thirty-two of the irradiated animals had had positive skin tests for tuberculosis and had received 250 mg. of streptomycin and 59 mg. of isoniazid intramuscularly twice a week (November 2, 1953 to January 15, 1956). All of these animals were being studied to observe the latent effects of ionizing radiation or nitrogen mustard. None of the 275 animals in the Randolph colony had been utilized in the experi-

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mental program of the Laboratory. Because the data on the Austin colony are more complete, only they are reported unless otherwise specified.

The animals varied in age from 3 to 9 years, and in weight from 2.5 to 10.0 kg. Seven of the 283 animals were female; only males are used in the experimental program of the laboratory. The animals were maintained on a daily diet consisting of approximately 114 gm. of monkey meal (whole wheat flour, 47 per cent; soy bean oil, 17.5 per cent; powdered milk, 5 per cent; bone meal, 5 per cent; molasses, 4 per cent; wheat germ meal, 4.5 per cent; cornmeal, 10 per cent; salad oil, 5 per cent; salt, 1 per cent; irradiated yeast, 1 per cent), supplemented with fresh fruit (oranges, grapefruit, apples, or bananas) and vegetables (carrots, lettuce, potatoes, or cabbage) three days per week. Because of the aforementioned error in food manufacture, the diet of the colony was altered so as to include 162,000 U.S.P. units of vitamin D per animal per day (Table I). Each also received approximately

TABLE I

*Analysis of Monkey Meal**

		A†	B†	C†
Total solids	Per cent	91.43	90.88	89.34
Moisture	Per cent	8.57	9.12	10.66
Protein	Per cent	22.19	19.42	20.17
Fat	Per cent	7.38	7.97	4.59
Ash	Per cent	6.92	7.83	5.16
Fiber	Per cent	2.81	2.85	2.40
Carbohydrate	Per cent	52.13	52.81	56.98
Calcium	Grams (per animal, per day)‡	1.54	3.50	0.87
Phosphorus	Grams (per animal, per day)	0.72	2.90	0.62
Vitamin D	U.S.P. units (per animal, per day)	4,252	163,000	40.00

* Analyses were performed by the Food Research Laboratory, Long Island City, N.Y., and by the Fourth U.S. Army Medical Laboratory, Fort Sam Houston, Texas.

† Diet A—Standard monkey diet fed prior to high vitamin D diet.
Diet B—High vitamin D diet.
Diet C—Diet fed subsequently; beginning on August 11, 1956, each animal received a vitamin supplement containing approximately 400 units of vitamin D daily.

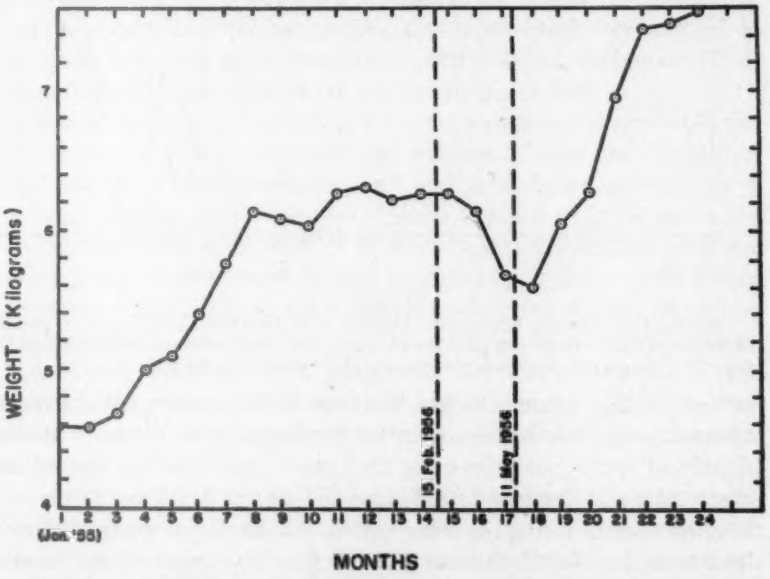
‡ Each animal received approximately 114 grams of the monkey meal per day.

3.5 gm. of calcium and 2.9 gm. of phosphorus per day. The animals in the Austin colony received the high vitamin D diet for 81 days (February 15 to May 7, 1956), and the animals in the Randolph colony for 86 days (January 23 to April 20, 1956) before the error was discovered and the diet was changed. On April 20, 1956, the animals in the Randolph colony, and on May 7, 1956, the animals in the Austin colony, were placed on a diet low in vitamin D. The amount of calcium and phosphorus in the diet was also reduced. In addition, each animal in the Austin colony received 50 mg. of Diamox (aceta-

zoleamide) and 4 cc. of aluminum hydroxide gel per day for 60 days in an attempt to maintain a slight acidosis. It was thought that this might tend to lessen the deposition of calcium in the tissues and promote its excretion. Antibiotic agents (penicillin, streptomycin, or chloromycetin) were used in the treatment of respiratory and gastrointestinal infections. A vitamin supplement was added to the diet on August 11, 1956, so that thereafter each animal would receive a total of 400 U.S.P. units of vitamin D daily.

The first clinical evidence of hypervitaminosis D was an increased incidence of upper respiratory infection and diarrhea occurring during late April and early May 1956. This was greater than was usually noted during this period of the year.

Each animal in the colony is weighed monthly. A graph of the average weight of 20 nonirradiated control animals over a period of 2 years, indicates the onset of weight loss shortly after the inception of the high vitamin D diet (diet B, Text-fig. 1). The weight loss con-



Text-figure 1. Weight of 20 nonirradiated control animals over a period of two years. The vertical lines represent the period the colony was on the high vitamin D diet. Note the weight loss during and shortly after that period.

tinued for approximately one month after diet B was terminated. Immediately following this, the entire group of animals gained weight progressively. A similar weight loss was also noted in the colony as a whole during the high vitamin D diet.

A peripheral blood count and smear are obtained on selected animals in the colony every 2 weeks to 1 month. Most of these animals had been irradiated. However, a group of nonirradiated control animals was available for study. An appreciable decrease in erythrocytes and hemoglobin during and after the period of excess vitamin D intake was evident (Text-fig. 2). When compared to the period immediately

Text-figure 2. Average hemoglobin values of 11 nonirradiated animals over a period of two years. The vertical lines represent the period the colony was on the high vitamin D diet. Note the drop in hemoglobin during and shortly after this period.

before the high vitamin D diet, the drop in hemoglobin noted over a 28-week period starting shortly after the beginning of the toxic diet is significant to the .001 level using the *t* test of the difference for paired observations. A decrease in red blood cell counts significant to the .01 level was noted during the same period. Similar hematologic findings have been described in human patients who have received high doses of vitamin D over long periods of time.[2]

Blood urea nitrogen (BUN), calcium, and phosphorus determinations on the serum of 40 monkeys were done within two weeks after the high vitamin D diet was terminated and repeated approximately 9 months after termination of the episode. The average BUN for the groups on the first examination was 26.3 (15 to 37) mg. per hundred milliliters and 9 months later was 19.4 (11 to 24) mg. When compared

to the average BUN of 10 "normal" animals, 19.8 (16 to 24) mg.,* this information suggests that a minor degree of renal failure was present in many of the animals near the end of the high vitamin D diet and that the renal function improved during the succeeding period of several months. The average serum calcium level initially (May 18, 1956) was 13.4 (11 to 17) mg. per hundred milliliters and the average serum phosphorus was 4.7 (3 to 7) mg. Nine months later, the average serum calcium value was 12.2 (10 to 14) mg., and the serum phosphorus, 6.8 (4 to 8) mg. This may be compared to values for calcium of 11.36 mg. and for phosphorus of 3.7 mg. given by Wats and Das Gupta[3] for "normal" *Macaca mulatta*. Similar elevations of BUN, calcium, and phosphorus have been described both in human subjects and in experimental animals receiving high vitamin D diets.[4-6]

Electrocardiographic tracings were done on 12 animals shortly after diet B was terminated. Two significant alterations were noted. In some, the T waves were rounded, a feature consistent with the effect of hypercalcemia. Large peaked T waves as seen with hyperpotassemia were also noted. Because of the very rapid heart rate natural in monkeys and their excitement from being handled, evaluation of an abnormality of the QT interval was extremely difficult.

Roentgenograms were made of the torso, including the proximal ends of the humeri and femurs in selected animals. These were obtained at the time the toxic diet was stopped, and at monthly intervals for 6 months. In the long bones no changes characteristic of prolonged vitamin D intoxication were seen. Soft tissue calcifications were not noted in the films, although, as will be shown later, they were observed in necropsy specimens obtained from animals dying during this period.

Catheterized urine specimens were procured from several animals immediately after the high vitamin D diet was terminated. These showed a large amount of sediment consisting chiefly of calcium phosphate crystals. Leukocytes, red blood cells, epithelial cells, and granular casts were also found.

Approximately a month after the high vitamin D diet was terminated, the surviving animals in the colony appeared to be in good health, and no symptoms which could be related to the high vitamin D diet were noted in the period that followed.

Pathologic Study

The following data are based on a study of 114 animals from the Austin colony that died or were sacrificed between June 20, 1955 and May 7, 1957. Twenty-six of these monkeys died between June 20, 1955

* Obtained from the Los Alamos Scientific Laboratories, Los Alamos, New Mexico.

and February 14, 1956, while the colony received the nontoxic diet A (Table I). These animals served as controls. Thirty-one died between February 15, 1956 and May 7, 1956, while the colony subsisted on diet B (high vitamin D). Fifty-six animals died while receiving diet C during the year following the discontinuance of the high vitamin D diet. Most of the tissue procured at necropsy from the 110 animals that died in the Randolph colony from the day that group of animals received diet B until one year later (January 23, 1956 to January 23, 1957) was also studied. The pathologic changes in the tissues from the two groups were very similar. Since the material from the Austin colony was more suitable, it only is used for this report unless otherwise noted.

All of the animals reported were necropsied and tissues were taken for microscopic examination from the parotid gland, submaxillary gland, esophagus, stomach, small and large intestine, pancreas, liver, larynx, trachea, lung, heart, aorta, kidney, urinary bladder, prostate, testis, adrenal, thyroid, pituitary, cerebrum, cerebellum, sternum, rib, vertebrae, head and shaft of the femur, lymph nodes, spleen, skin, and skeletal muscle. The eyes were examined in more than half and the parathyroid glands were available in more than three fourths of the animals. Tissues were fixed in buffered neutral formalin, embedded in paraffin, and sectioned at 6 microns thickness. Sections were stained with hematoxylin and eosin. The von Kossa method was used in demonstrating calcium in tissue.[7] Von Kossa stains were prepared on sections of the kidney, heart, and lung of all the animals reported. In 14 animals dying at the height of the vitamin D feeding episode, von Kossa stains were done on all the tissue. The alizarin red method for staining calcium[7] was applied to occasional tissues. Because the distribution of calcium was shown to be similar by the two methods, the alizarin red method was not used on all tissues. Gomori's method[8] for demonstrating iron in tissue was used regularly.

Results

The characteristic lesions found in the animals with hypervitaminosis D consisted of mineral deposits with or without associated inflammation, depending on the tissue involved. The term "mineral deposits" is used since calcium and iron were found in all of these lesions, and phosphorus was probably present in most (Table II). Other minerals may have been present, but no attempt was made to demonstrate them. In addition, an organic component was present in the lesions,

particularly in the kidneys. This consisted of an amorphous, eosinophilic material which was positive with the periodic acid-Schiff (PAS) stain, metachromatic with toluidine blue and stained blue with Alcian

TABLE II

*Chemical Analysis of Kidney, Heart, and Lung for Calcium and Phosphorus**

Organ	Control animals			Hypervitaminosis D animals		
	Animal number	Calcium†	Phosphorus†	Animal number	Calcium†	Phosphorus†
Kidney	112+	2.63	2.90	V-5	10.16	3.03
	K-8	1.46	2.90	370+	8.83	4.53
	309+	3.10	2.30	Z-5	19.67	7.00
	127	0.96	6.00	116	6.00	10.80
	489	2.40	7.08	133	12.00	9.60
	686	0.48	8.76	61+	6.72	7.80
Average		1.84	4.99		10.56	7.13
Heart	112+	0.24	4.92	V-5	1.97	2.63
	K-8	1.80	0.97	370+	3.30	4.67
	309+	5.28	4.80	Z-5	1.20	3.00
	127	3.60	4.44	116	5.28	8.64
	489	3.84	4.20	133	4.44	4.56
	686	2.88	5.76	61+	2.16	4.92
Average		2.94	4.18		3.06	4.74
Lungs	112+	2.97	2.23	V-5	4.57	2.43
	K-8	1.43	2.20	370+	3.60	2.30
	309+	3.60	2.60	Z-5	2.40	6.96
	127	5.78	7.92	116	5.28	8.64
	489	2.46	6.48	133	3.84	6.00
	686	3.60	6.00	61+	6.96	2.40
Average		3.31	4.57		4.44	4.79

* All determinations were done at the Fourth U.S. Army Medical Laboratory, Fort Sam Houston, Texas, under the supervision of Capt. Arthur C. Dixon.

† All values given as mg. of calcium or phosphorus per 100 mg. of dried tissue.

blue. These characteristics suggest that this material contained an acid mucopolysaccharide. A similar material has recently been described in the soft tissue lesions of rats receiving high doses of vitamin D.[9]

The livers of two animals dying within one month after diet B was terminated, were assayed for vitamin D and compared to similar assays on two control animals and two animals which died 9 months after the termination of diet B (Table III). The vitamin D content in the first two animals was high as compared to the controls. The concentration of vitamin D in the last two animals was near the control level.

Urinary Tract

Characteristic lesions were found more consistently in kidneys than in any other tissue. An animal which died 28 days after the beginning of diet B was the first to show focal mineral deposits. The kidneys of

TABLE III
*Analysis of Liver for Vitamin D**

Animal No.	Group 1†		Group 2‡		Group 3§	
	a	b	1	2	167	639
U.S.P. units of vitamin D per gm. of fresh liver	3.5	1.4	11	10	2.1	4.2
Average	2.45		10.5		3.15	

* Bio-assays performed by Food Research Laboratories, Inc., Long Island City, N.Y.
† Two "normal" animals obtained from Okatie Farms, Hardieville, S.C.
‡ Two animals dying within one month after diet B was terminated.
§ Two animals dying nine months after the termination of diet B.

every animal that died thereafter manifested some degree of mineralization.

The earliest lesions (28 to 54 days) consisted of occasional small deposits in the tubules, often in the basement membranes. Many were not associated with an inflammatory reaction. The number of deposits increased with time so that by the 76th day, severe lesions were noted (Fig. 3). In these cases the deposits could be seen on gross examination as yellowish-white flecks in the cortex and medulla (Fig. 1). They could also be seen in postmortem roentgenograms of the kidneys (Fig. 2).

Microscopically, the deposits appeared in the tubules for the most part or in the surrounding interstitial tissue. The distal convoluted, proximal collecting, and thin loop tubules were involved most frequently. The proximal convoluted tubules and medullary collecting tubules were relatively spared. In the sections stained with hematoxylin and eosin, the deposits varied from a dark or light blue to an almost colorless appearance. Calcium and iron were demonstrated consistently in them. Foreign body type giant cells and mononuclear cells were often found in and about the deposits, and polymorphonuclear cells were noted occasionally (Fig. 4). Many of the lesions were metachromatic when stained with toluidine blue. They were also PAS positive and stained blue with Alcian blue before and after decalcification. In several cases, deposits were noted in the glomeruli and also in the walls of small arteries and veins. Hyaline casts were often present in the tubules. In some instances, the tubules above the

TABLE IV

Distribution of Calcium Deposits in Heart, Kidneys, and Lungs

Organ	Animal number									
Heart	213+	551	54+	C-8	55+	X-7	133	61+	86+	165+
Left ventricle										
Apex	++*	o	+	++++	++	+	+	†	+	o
Base	+++	+	o	+++		+	o	+	+	
Middle	++++	++	+	++++	++	+	+	+	+	o
Right ventricle										
Apex	o	o	o	+++	o	o	o	o	o	o
Base	+	o	o	++	o	o	o	o	o	o
Left atrium	+	o	o	++	++	o	o	o	o	o
Right atrium	o	o	o	o	o	o	o	o	o	o
Kidney										
Superior pole										
Right	+++	+++	++	++	+++	+++	+++	+++	+++	+
Left	+++	++++	++	++	+++	+	+++	+++	+++	+
Inferior pole										
Right	+++	+++	+++	++	+++	+++	+++	++	+++	+
Left	+++	++++	+++	++	+++	+	+++	+++	+++	+
Middle										
Right	+++	++++	+++	++	+++	+++	+++	+++	+++	+
Left	+++	+++	+++	++	+++	+	+++	+++	+++	+
Lung										
Upper lobe										
Right	++++	+++	+++	++	++	++	+	++	+	o
Left	++++	+++	+++	++	++	++	+	++	+	o
Middle lobe										
Right	+++	+	++	++	++	++	+	++	+	o
Left	++++	+	++	++	++	+	+	++	+	o
Lower lobe										
Right	++++	++	++	++	++	++	+	++	+	o
Left	++++	++	+++	++	++	++	+	++	+	o

* The quantity of calcium was estimated on the basis of the amount of black precipitate in the von Kossa stained sections: ++++ = most severe calcification noted in a given organ, +++, ++, + = 75, 50, 25 per cent of the maximum.

† Tissue not available for examination.

mineral deposits were dilated. Multiple sections were taken from both kidneys of 10 animals dying at the height of the vitamin D feeding episode. Although there was some variation in the number of calcium deposits from slide to slide, no one portion of the kidney was more consistently involved than another (Table IV).

The degree of calcification in the kidneys seemed to decrease about a month after diet B was terminated and continued to do so throughout the remainder of the year. In addition to a decrease in the number of deposits, there was a decrease in the amount of inflammatory reaction as well. For the last half of the year, no inflammation was noted in relation to the renal deposits. One animal dying in the Randolph colony approximately 6 months after the end of the high vitamin D diet showed renal lesions very closely resembling those found in human patients with malignant hypertension. As this was the only animal in which such lesions appeared, it is difficult to relate them to the high vitamin D diet.

The renal pelvis and bladder and the testes were not abnormal nor were stones found in the urinary tract. In one animal, fine granular deposits of calcium were noted in the stroma of the prostate in relation to the elastic fibers, and in two cases small deposits were found in the epithelium lining the seminal vesicles.

Respiratory System

The lungs were often affected but not as regularly as the kidneys: mineral deposits were found in the lungs of 23 of the 39 animals dying between the 55th to 208th day. The early lesions appeared in the mucosa of the small bronchi and alveolar ducts where mineral deposits frequently lay within the basement membranes. The lesions contained both calcium and iron and were often surrounded by mononuclear and foreign body giant cells. The granulomas caused deformity of the overlying epithelium in some cases and occasionally resulted in ulceration. In the animals dying near the 81st day, the deposits were more extensive. In these, the alveolar walls were incrusted with calcium and iron (Fig. 5). Also multiple small granulomas containing calcium and iron were found in the mucous membranes of the bronchi, trachea, and larynx, on and around the basement membranes. Mineral deposits were not found in animals dying in the Austin colony after the 281st day. However, 3 animals from the Randolph group that died almost one year after diet B was terminated showed extensive calcifications. Some of the deposits occurred in the lumens of the alveoli.

In the older control animals, the laryngeal cartilages were often calcified, but this was not the case in the bronchi. In the animals that

received the high vitamin D diet, the bronchial cartilages were frequently calcified. The first animal to show this, died on the 68th day. Twenty of the 29 animals dying between the 68th and 230th days had some calcification of the bronchial cartilages. Presumably this was related to the high vitamin D diet.

Cardiovascular System

Cardiac lesions were noted only in the animals dying between the 55th and 86th days: 9 of the 24 animals dying during this period showed lesions. Abnormality was recognized grossly in all 9 animals. This consisted of a scattered grayish-yellow mottling of the myocardium (Fig. 6). Microscopically, the lesions were focal and exhibited calcium and iron deposits. The deposits consisted of fine granules or irregular plaques along the muscle fibers. Evidence of myofibril degeneration and necrosis was found in these areas (Fig. 8). Mononuclear cells also were often found, although the inflammatory reaction was not marked. Foreign body giant cells were seen only occasionally. In the animals with minimal myocardial damage, the lesions were predominantly subendocardial in location. In the more severe cases, lesions were found throughout the myocardium of the left ventricle (Fig. 7). In these cases, intramyocardial vessels were often mineralized, sometimes partially necrotic and occasionally the seat of thrombosis. Multiple sections of the heart in the 9 cases showed that the lesions were always in the left ventricle, occasionally in the left atrium and right ventricle, but never in the right atrium (Table IV).

Focal areas of scarring and chronic myocarditis were found in the left ventricle of 2 animals dying almost a year after the termination of the high vitamin D diet. Although no calcium was seen in these lesions, their distribution suggested that they might represent the residuals of myocardial damage similar to that seen in animals dying between the 55th and 86th days.

Mineral deposits were also found in the aortas of 12 of 34 animals dying between the 55th and 140th days. The deposits were predominantly in the media along the elastic fibers. The lesions were not seen on gross examination and there was no loss of aortic elasticity. In animals from Randolph Field, gross aortic calcifications were found twice, and in the aorta from a third case, a calcified area was surrounded by a dense infiltration of neutrophils. This represented the sole instance of aortic mineralization associated with inflammation.

If mineralization of small vessels in the presence of massive parenchymal calcification be excluded, disseminated lesions of small arteries in soft tissues were encountered in only 3 animals: one animal from

the Randolph Field colony and one from the Austin colony died near the end of the high vitamin D diet. The third animal was from the Randolph group and died 11 months after the termination of diet B. The deposits lay along the internal elastic laminas in all cases (Fig. 9).

Digestive Tract

Next to renal involvement, the earliest and most common evidence of calcification was found in the salivary glands: 27 of 43 animals dying between the 47th and 226th days had these lesions. The submaxillary glands were involved almost twice as commonly as the parotid glands. The deposits appeared in the walls or lumens of small ducts (Fig. 10). Duct dilatation was found occasionally, in both the presence and the absence of demonstrable ductal obstruction.

Mineral deposits were noted in the mucosa of the stomach of 10 animals. In most cases they were scanty, but in one they were extensive (Fig. 12). In the latter, the muscularis mucosae was involved but not the other muscle layers. The mineral deposits were often associated with foreign body granulomas. Similar mineral deposits were noted in the jejunum of one animal and the colons of 2 animals. In all 3 cases, the deposits were slight and not associated with an inflammatory reaction.

Lesions attributable to hypervitaminosis D were not found in any of the sections taken from the tongue, esophagus, liver, and pancreas.

Central Nervous System

Several months after the termination of diet B, 6 animals which had received high doses of x-ray irradiation to the head died. Necrotic areas were found in the path of irradiation through the brain. Calcium and iron deposits were found in these. Similar deposits have been noted in monkeys receiving focal ionizing irradiation to the head in the absence of a high vitamin D diet.[10] Other evidences of calcification were not noted in the central nervous system.

Endocrine Glands

Although sections were examined from the pituitary, thyroid, and pancreas in all cases, no mineral deposits were noted in these areas. Laminated deposits were frequently found in the inner portion of the adrenal cortex (Fig. 11), but these were present equally frequently in the control animals.

Although the parathyroid glands were not weighed, they did not appear to be enlarged. They were composed characteristically of uniform cells with dark-staining nuclei and scant cytoplasm. The average

diameters of 50 cells from 4 control animals and 4 animals dying at the height of the vitamin D episode were compared. There was no significant difference between the 2 groups as has been described in the dog.[11]

Musculo-skeletal System

There was little evidence of destruction in the sections of bone examined. In one animal the lacunas in the vertebrae were larger than those seen in other cases; however, there was no evidence of an increase in the number of osteoblasts, nor was there any gross evidence of demineralization. The paucity of bone lesions in these animals may be due to the high calcium and phosphorus content of the diet. The bone marrow in a number of cases was atrophic, although this could be related either to high doses of whole-body ionizing radiation or to the administration of nitrogen mustard. Mineral deposits were not noted in the sections of skeletal muscle which were usually taken from the sartorius muscle.

Other Tissues

Although sections were examined from the skin, testes, spleen, lymph nodes, and occasionally from the eyes, mineral deposits as perceived in the other soft tissues were not found.

Discussion

Since hypervitaminosis D was first noted in 1928, it has been described in a number of species, including man.[12-14] Only two reports were found in the literature dealing with this condition in monkeys. Hess and Lewis[15] produced it in monkeys but they were interested primarily in the blood calcium and did not describe the pathologic lesions. The 19 monkeys reported by Cowdry and Scott[16] apparently received more vitamin D than our animals. Despite this, the mineral deposits in the soft tissue were minimal. The high calcium and phosphorus content of the diet of our animals may explain the extensive deposits found in the soft tissue of many.

The quantity of vitamin D received by the animals included in this report was well above the toxic dose. Despite this, 28 days elapsed before any pathologic lesions attributable to hypervitaminosis D appeared in the tissues. It was 55 days before really severe lesions were noted. The alterations were most severe in animals dying about the time the high vitamin D diet was terminated and for one month thereafter. The amount of calcification and the number of organs affected decreased with time, so that animals dying one year after the termination of diet B showed slight mineralization. The animals dying in the Randolph Field colony generally revealed more severe lesions. This

was particularly evident in the necropsy examination of animals during the last half of the year following the termination of diet B. Three animals from the Randolph group during that period had extensive mineral deposits in the lungs, as well as in the kidneys. Two factors probably contributed to the difference in the two groups: first, the Randolph group was on diet B for 5 days longer; second, the Randolph group was housed outdoors and was thus exposed to the sun, whereas most of the Austin colony was maintained indoors.

The distribution of the lesions in these monkeys was similar to that described in other species. However, the degree of vascular involvement seemed to be less than that described in rodents.

The reaction of the tissues to the mineral deposits varied considerably. The deposits in the kidneys, mucosa of the stomach, and the mucosa of the respiratory tree usually were surrounded by mononuclear cells and foreign body giant cells forming small granulomas which distorted the tissues. On the other hand, there was usually no inflammatory reaction to the mineral deposits in the aorta and salivary gland. The muscle fibers in the heart containing mineral deposits showed evidence of degeneration or were frankly necrotic. A mononuclear cell reaction was frequently seen in the myocardium but was usually mild, and foreign body giant cells were observed only occasionally.

The reports in the literature dealing with the pathologic alterations of hypervitaminosis do not mention the presence of inorganic iron in the lesions as observed in our animals. The presence of iron in the mineral deposits raises the possibility that there was systemic disturbance of iron metabolism in addition to that of calcium and phosphorus. This is not likely, for as Bunting[17] has pointed out, iron is usually found in soft tissue calcifications regardless of the cause of the lesions.

Mulligan[18] has suggested that the tissues where acids are formed, are rendered alkaline when these acids are secreted so that calcium deposition is favored. The high incidence of calcification of the acid-secreting kidneys, mucosa of the stomach and lungs, and the absence of calcification of the alkaline-secreting pancreas and liver in our animals adds support to this idea. The tendency for the calcifications to be greater in the left ventricle and atrium than in the right ventricle and atrium, as noted in our animals and as has been reported in other species, may be explained on the same basis; that is, the pH of the arterial blood is higher than the venous blood.

Most of the animals reported on here which died spontaneously, succumbed as a result of acute infection of the lungs or colon. Though the toxic amounts of vitamin D may have lowered the resistance of the colony to infection, it is not unusual to have monkeys in the colony

die of pneumonia or acute colitis, particularly in the spring. Some of the animals apparently died of hypervitaminosis D. In these, the calcification and degeneration in the myocardium were extensive, and there was evidence of congestive heart failure: dilatation of the ventricles, congestion and edema of the lungs, and congestion of the liver. The renal lesions in a number of the animals were extensive, and renal failure was probably a lethal factor. It is difficult to evaluate this, as studies of renal function were not done on any of the animals that died. Blood urea nitrogen determinations were done on 40 animals which did not die, and the levels were elevated in many.

Because many of the animals had received some type of ionizing radiation or nitrogen mustard, the relationship to hypervitaminosis of some of the pathologic lesions found at necropsy was doubtful. Hypoplasia of the bone marrow, atrophy of the spleen, lymph nodes, and thymus are features attributable to radiation or nitrogen mustard administration and have not been described in hypervitaminosis D in other species. When these lesions were found in our animals, they therefore could be related in every case to either ionizing radiation or the administration of nitrogen mustard. On the other hand, widespread soft tissue mineralization accompanied by inflammation or degeneration is not an anticipated reaction to irradiation or nitrogen mustard. When appearing in animals receiving a diet high in vitamin D and not in the control animals, this obviously reflects the effect of the high vitamin D diet.

Summary

A colony of 558 monkeys (*Macaca mulatta*) was inadvertently given a diet high in vitamin D for a period of approximately 3 months. The clinical and pathologic findings noted in these animals during the period of the high vitamin D diet and for a year thereafter have been described, and compared to those reported in other species. There were weight loss, anemia, elevation of blood urea nitrogen and serum calcium, and an increased incidence of diarrhea and upper respiratory infections during the period of acute exposure. Lesions were most common in the kidney, salivary gland and lung, and consisted of calcium and iron deposits which were often associated with a foreign body type reaction. The animals examined a year after the high vitamin D diet was terminated showed few lesions.

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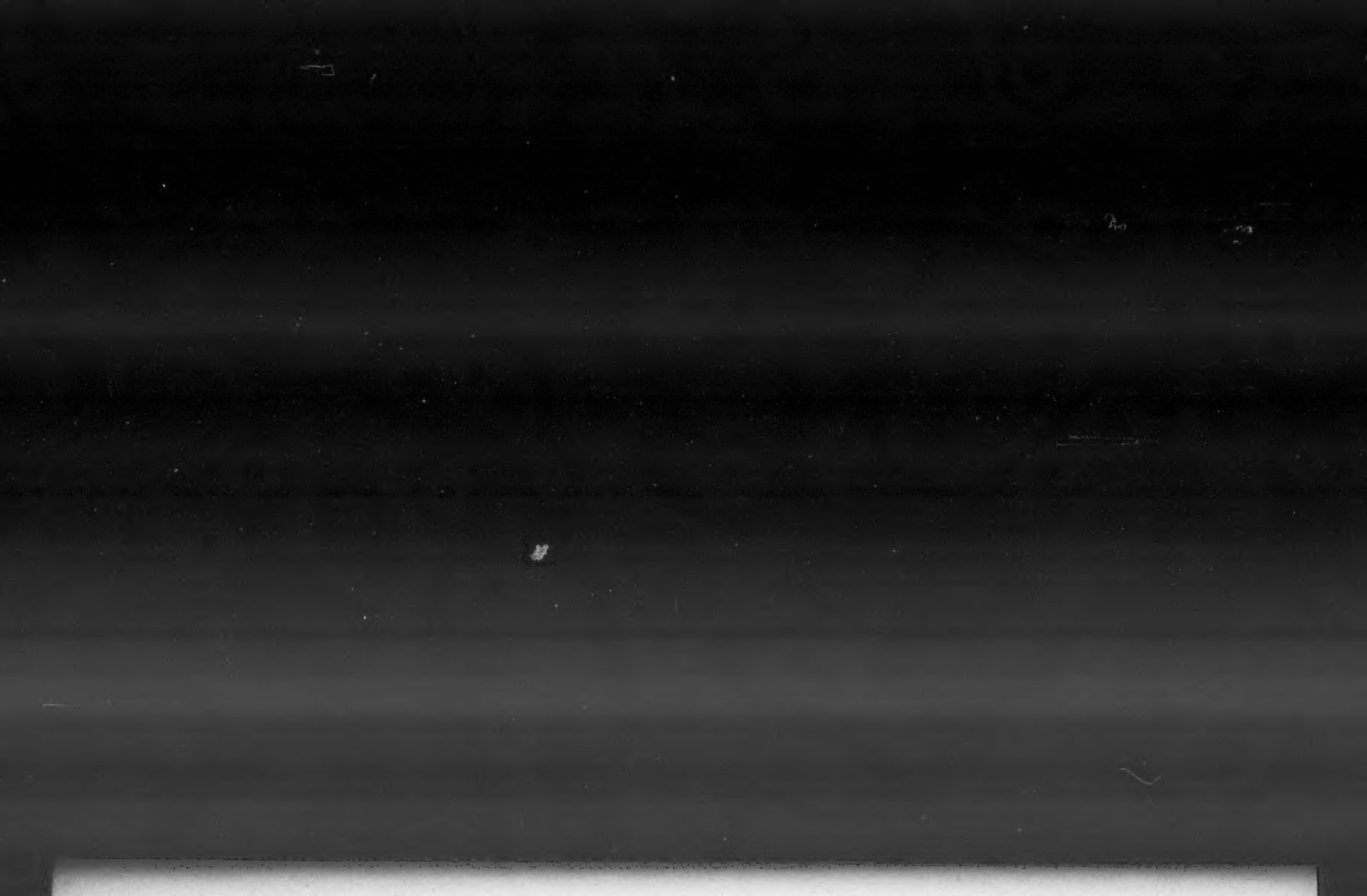
LEGENDS FOR FIGURES

FIG. 1. Kidney. White flecks of calcium are evident, particularly in the cortex. × 2.5.

FIG. 2. Postmortem roentgenogram of the kidney. Note the small opacities throughout the kidney. × 2.5.

FIG. 3. Kidney. Mineral deposits are widely distributed. Von Kossa stain. × 6.



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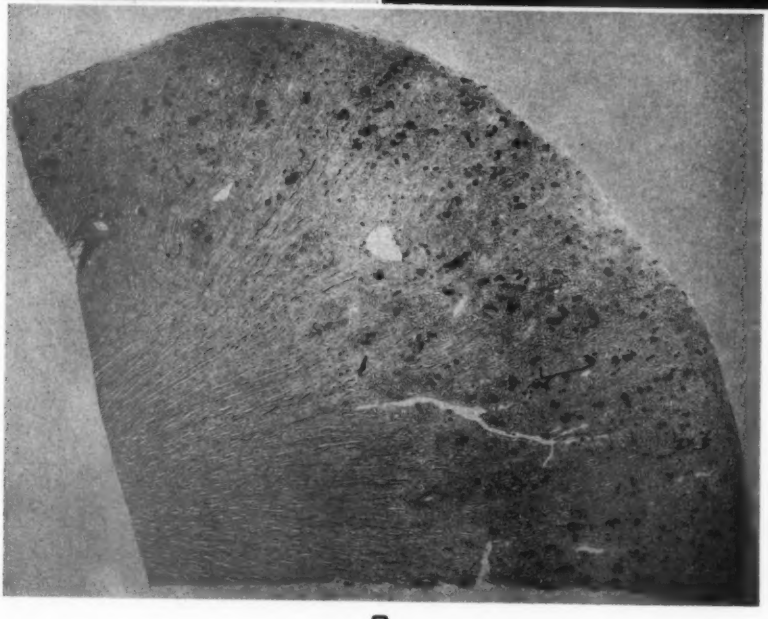

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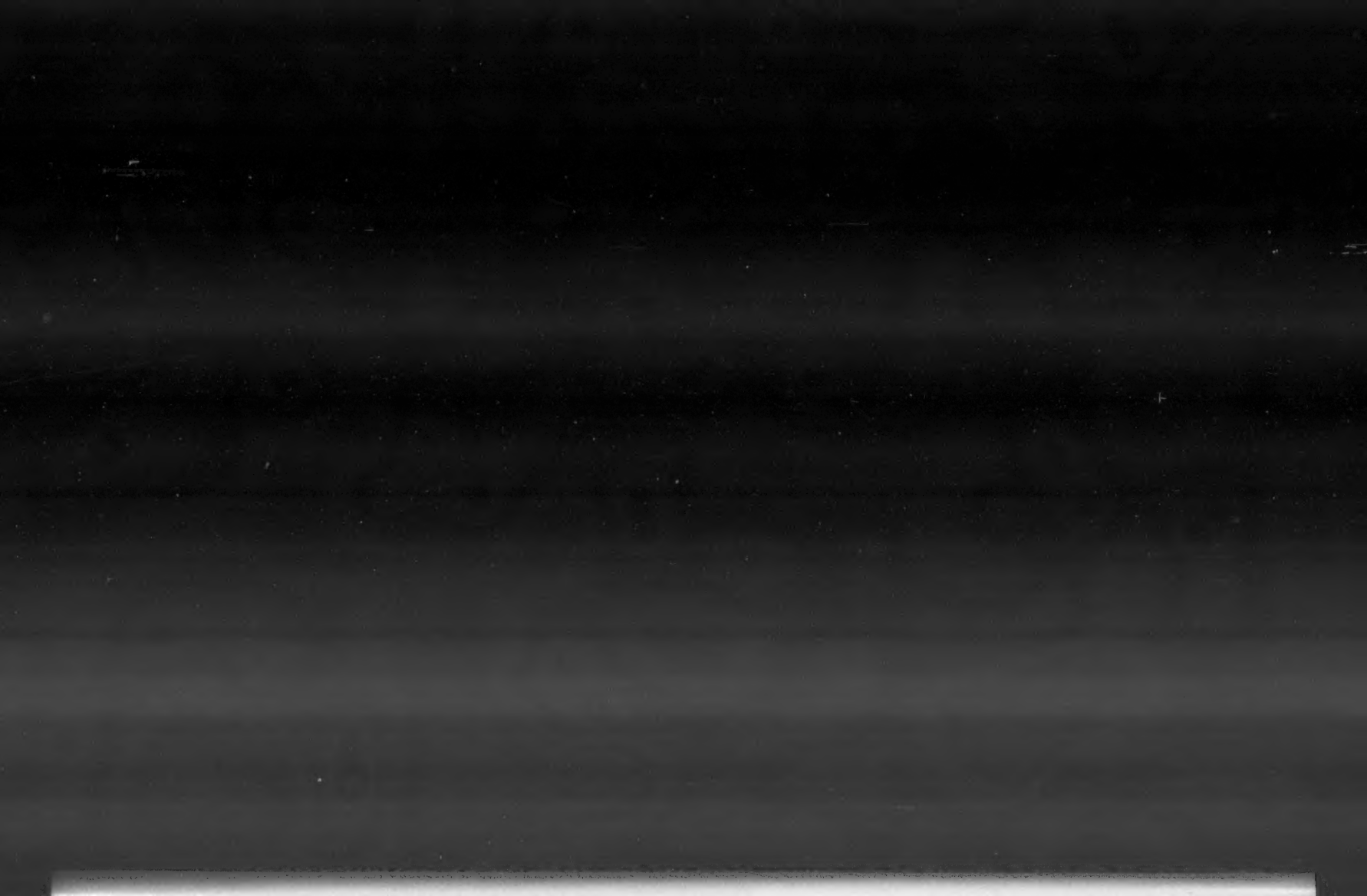
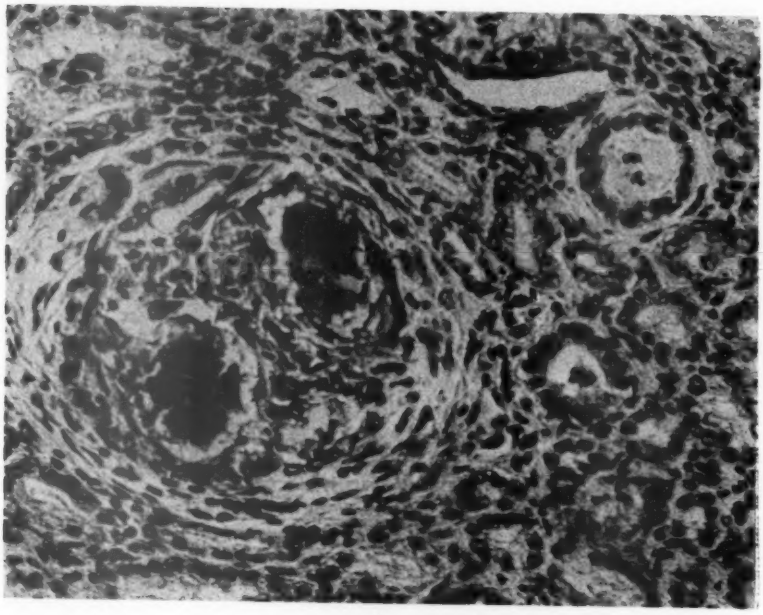
FIG. 4. Kidney showing granulomatous reaction to the mineral deposits. Hematoxylin and eosin stain. × 250.

FIG. 5. Lung showing extensive calcium deposits. Von Kossa stain. × 6.





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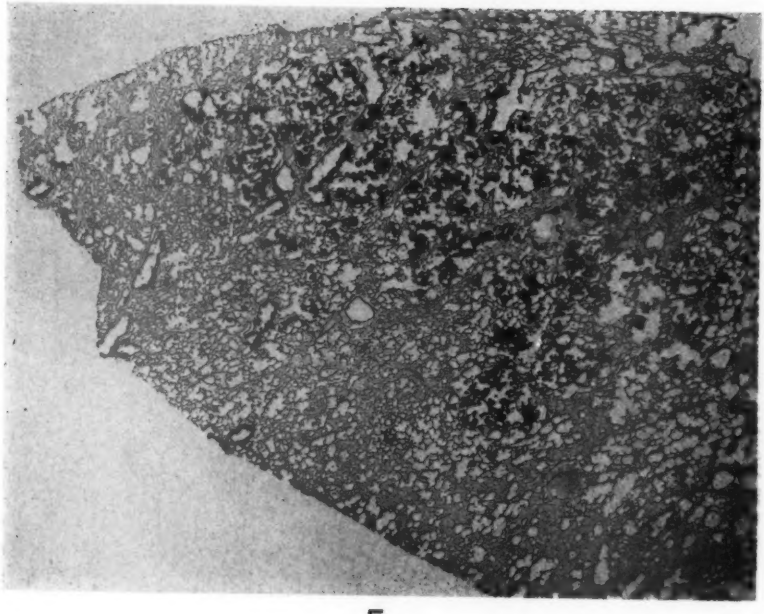

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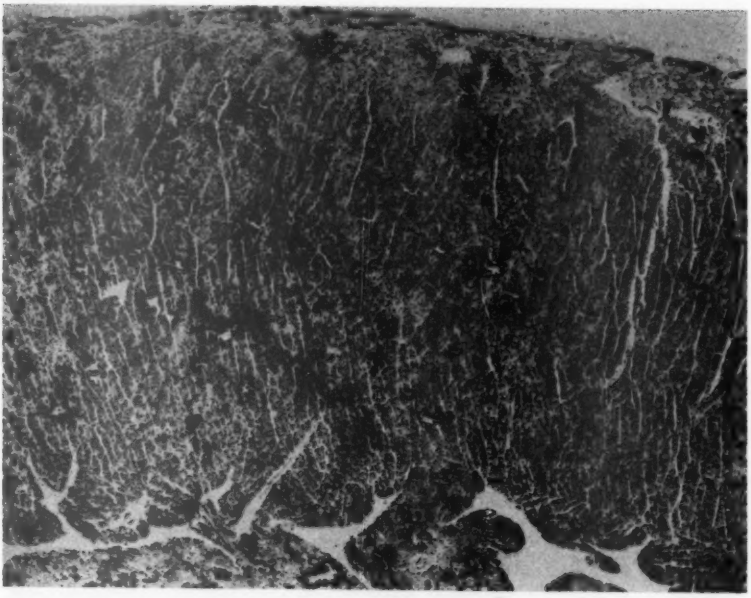
FIG. 6. Heart. Note the mottled appearance of the myocardium. × 2.5.

FIG. 7. Heart. Extensive calcium deposits are found throughout the myocardium. Von Kossa stain. × 12.

FIG. 8. Heart showing calcification and atrophy of muscle fibers. Hematoxylin and eosin stain. × 93.



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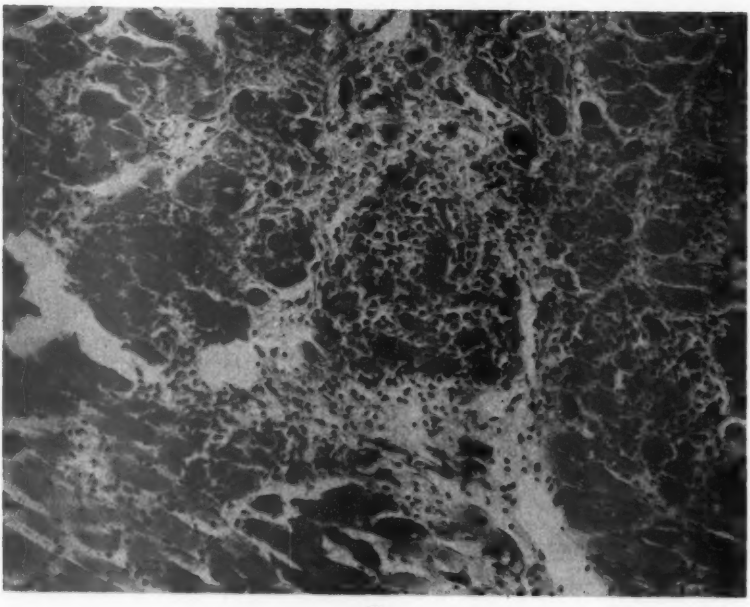

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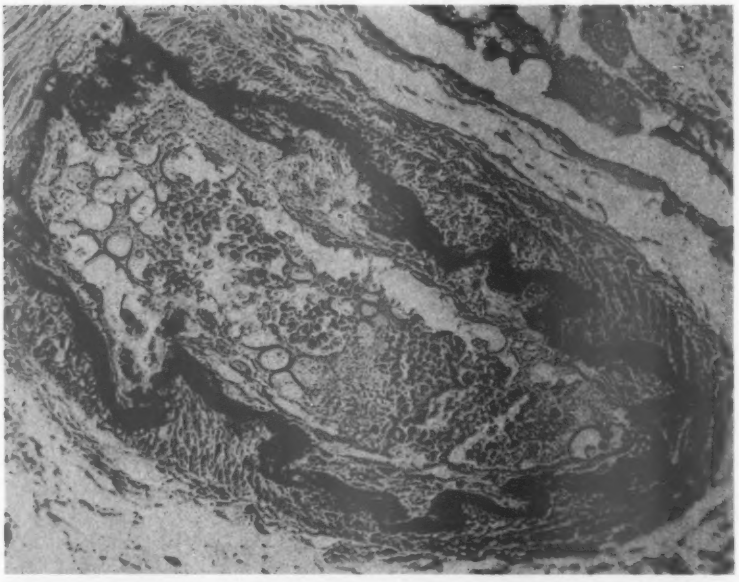

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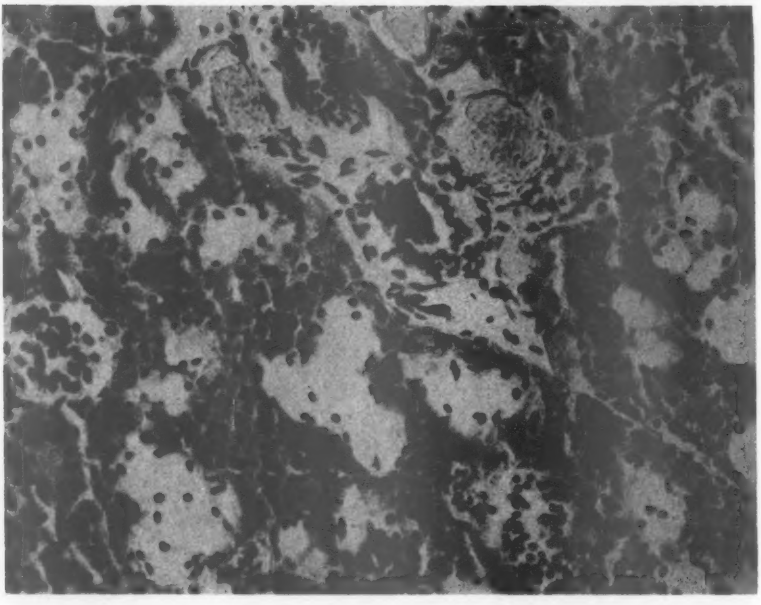
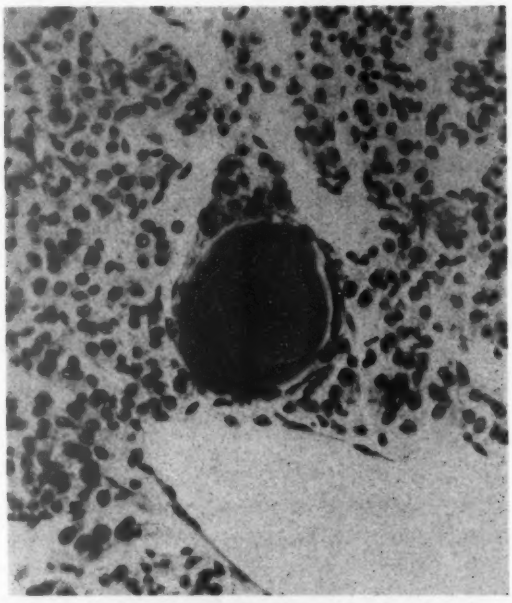


FIG. 9. Small artery from tissue adjacent to an axillary lymph node. The internal elastic lamina is calcified. Von Kossa stain. × 220.

FIG. 10. Submaxillary gland. A mineral deposit is found in a small duct. Hematoxylin and eosin stain. × 150.



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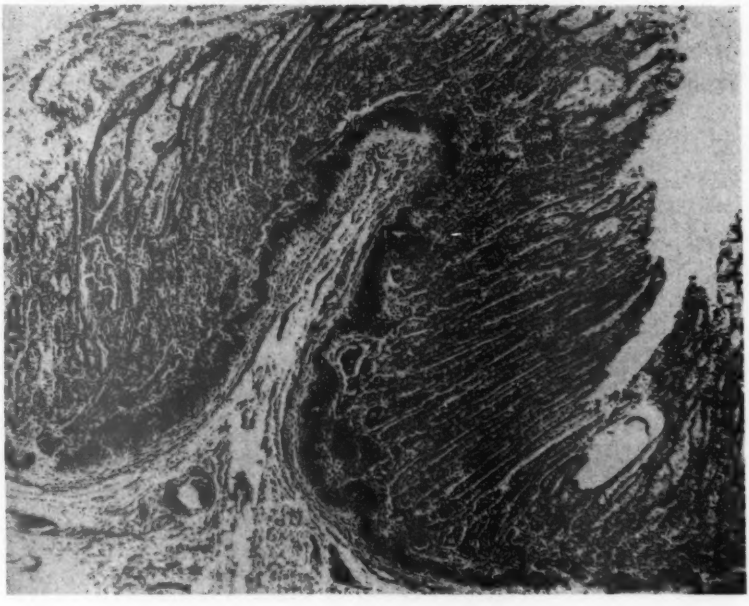

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FIG. 11. Adrenal. A calcified mass in the inner portion of the cortex. Hematoxylin and eosin stain. × 60.

FIG. 12. Stomach. Note the extensive calcification in the mucosa. Von Kossa stain. × 80.

SYSTEMIC EFFECTS IN RABBITS RECEIVING INJECTIONS OF PAPAIN AND CHONDROITIN SULFATE*

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The collapse of rabbit ears following intravenous injection of crude papain originally observed by Thomas,[1] has been found to be associated with the escape of an acid polysaccharide from the cartilage matrix into interstitial perichondrial spaces, lymph vessels, and regional lymph nodes.[2] Several observations and considerations indicate that this acid material is chondroitin sulfate. In this report the histologic changes in kidney, liver and blood, following papain injection, are described and compared with different manifestations resulting from the injection of commercially available chondroitin sulfate.

Methods

Rabbits treated with crude papain as previously described[1,2] were sacrificed at 7 hours and at 1, 3, 9 and 22 days following injection and necropsies were performed immediately.

Specimens from these animals and three controls were fixed in one or more solutions including: acetic alcohol formalin (A.A.F.), Carnoy's mixture, Bouin's fluid and calcium acetate formalin. The fixatives were prepared and a variety of staining procedures were carried out as summarized by Lillie.[3]

Results

After papain, sections of the A.A.F.-fixed tissue stained with azure A at pH 5.0 revealed frequent clumps of finely to coarsely granular, globular or fibrillar orthochromatic material in the lumens of many small to large arteries and veins throughout the body. Sections of clotted blood from these animals similarly fixed and embedded in paraffin showed comparable dark blue staining aggregates (Fig. 1). However, when stained with azure-eosin mixture, these bodies were eosinophilic. They stained red with the periodic acid-Schiff (PAS) procedure for polysaccharide and pink with the ninhydrin Schiff method for amino groups. They did not stain with the Hale dialyzed iron method for acid mucopolysaccharides.

Although fine and occasionally coarse orthochromatic granules are

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seen infrequently in vessels of normal rabbits, no aggregates morphologically similar to those illustrated have been found in these animals. The abnormal polymorphic material was found in abundance in the blood of rabbits 7 hours and 1 and 3 days after the administration of papain. Aggregates were seen occasionally in small hepatic and renal arcuate vessels at 9 days and in a very few renal vessels at 22 days.

The type of fixative utilized influenced both the morphologic and tinctorial appearances of the aggregates. The morphologic features appeared most conspicuously after A.A.F. fixation. With formalin fixatives (except Bouin's) there was clear distinction between the orthochromatic staining of the blood clumps and the metachromatic staining of the material previously described in lymph vessels.[2] In tissue fixed in Bouin's or Carnoy's fluid this distinction disappeared because the blood material also stained metachromatically (Figs. 2, 10, 11). With these two fixatives normal animals showed no blue or purple material in the blood so that there was greater contrast between unstained material in control animals and the metachromatic aggregates in those receiving papain.

Electrophoretic analysis of the plasma from a rabbit one day after the injection of papain revealed a strongly anionic abnormal component migrating far ahead of the normal serum proteins (Fig. 3). The appearance of the acid component coincided with a comparable diminution in the basic portion of the pattern; i.e., the β globulin plus fibrinogen peak.

Dried plasma smears of the experimental animals differed in an unusual and as yet unexplained manner from those of controls. They had the opaque whitish appearance of frosted glass as compared with the translucent normal smear. Viewed under the phase contrast microscope the frosty smear showed irregularly rounded vesicular bodies not seen in normal plasma smears (Figs. 5 and 6).

Severe tubular hemorrhage occurred in the kidney after papain administration. This involved the straight segments principally, and largely although not completely spared the convoluted portion of the proximal tubules (Fig. 7). Collecting tubules and glomeruli showed no hemorrhage. Distal tubules frequently were found to enclose fused metachromatic casts (Fig. 8). Animals sacrificed 1 to 3 days after the introduction of papain showed these lesions.

A procedure has been developed for demonstrating more effectively and for localizing hemorrhage in tissue fixed in Bouin's fluid. The method involves staining with azure A in the pH 2.5 to 3.5 range after only partial removal of the picrate by a 1 to 2 minute rinse in running water. This short rinse replaces the conventional thorough 30 minute

rinse to remove all picrate. It is assumed that in this procedure, picric acid is not removed by the brief rinse and forms an addition compound[4] with a blood constituent. This, being strongly acid, reacts with azure A at low pH. The dye colors the blood in vessels a dark reddish purple against a pale blue or colorless background and stains the blood in the tubules even more conspicuously. Sections stained by this method demonstrate the extent of the tubular hemorrhage more clearly and reveal its localization principally in the outer medulla (Fig. 9).

There was marked variation in response to papain. In less affected animals only the small vessels of the renal medulla contained the aggregates of abnormal material. Thus 2 of the 3 rabbits examined at 24 hours showed large amounts of material in vessels of all organs examined. The third animal (the one used for the electrophoretic analysis of plasma) was less affected in that the cartilage lost only part of its metachromasia, no hemorrhage occurred in the renal tubules, and a loss of liver glycogen was limited to the portal zones. The vessels in this animal did not reveal abnormal material except in the small vessels of the renal medulla, which contained numerous globular aggregates. Moreover, the more severely affected animals showed the greatest amount of material in these vessels, those of the papilla being filled particularly (Figs. 10 to 12). These vessels often appeared occluded by the foreign matter. In fact, in the rare fortunate instances where the section followed the course of an involved vessel, embolic plugging of the lumen was indicated by erythrocyte engorgement proximal to the obstruction (Fig. 13). Although the aggregates in blood vessels in A.A.F.-fixed material were orthochromatic elsewhere, they occasionally stained metachromatically in the renal *arteriolae rectae*.

In the animals examined 3 and 9 days after receiving papain, renal tubule casts were seen in the outer medulla. After neutral formalin fixation these stained green with pH 5 azure A and red with the PAS method. From their structure and the absence of associated fresh hemorrhages, these appeared to represent a phase of resolution of the hemorrhagic lesion (Fig. 14). The collecting tubules at 9 days contained PAS-positive, azure A nonreactive casts (Fig. 15), contrasting with the infrequent, weakly PAS-positive casts seen in control animals. In the animal examined at 22 days the tubules appeared normal.

Liver glycogen, characterized as the diastase digestible, PAS-positive, cytoplasmic constituent, was absent in the periportal regions in moderately affected animals (Fig. 16), and was altogether absent in severely affected animals 24 hours after papain administration. However, there was periportal glycogen depletion at 11 hours and complete

depletion at 24 hours in fasted controls. It may be significant, however, that the decrease of the periportal glycogen persisted in the animals examined 9 and 22 days after the introduction of papain. Moreover, in all the papain-treated rabbits, the liver cell cytoplasm, particularly in the periportal regions, stained in basophilic fashion with azure A (Fig. 18). The liver parenchymal cells of one rabbit which succumbed 20 hours after the intracardiac administration of papain contained numerous, dense, orthochromatic, PAS-positive bodies, usually seen in vacuoles.

Two rabbits given intravenous injections of 3.0 gm. of chondroitin sulfate prepared from pig nasal cartilage (General Biochemicals, Inc.) and two rabbits treated similarly with chondroitin sulfate derived from beef tracheal and nasal cartilage (Nutritional Biochemicals Corp.) were sacrificed 4 and 5 hours later respectively. Plasma smears were prepared from an animal treated with beef chondroitin and were found not to reveal a frosty appearance upon drying. Tissues fixed in the battery of fixative solutions showed no polymorphous basophilic material in blood vessels and no renal hemorrhages. However, after the introduction of pig chondroitin, when fixed with A.A.F. or Bouin's fluid, the epithelium of the proximal and distal straight renal tubules exhibited metachromatic staining (Fig. 19). Only a few isolated tubules stained metachromatically in animals treated with beef chondroitin. After the introduction of beef chondroitin and with Bouin's fluid fixation, the renal cortex revealed very numerous dark red PAS-positive globules, 1 to 6 μ in diameter in glomerular and postglomerular capillaries, in Bowman's capsular spaces and in the proximal convoluted tubules at their points of origin (Figs. 20 and 21). In control animals similar bodies were seen in Bowman's capsular spaces but were much less abundant. Medullary capillaries contained fewer such bodies; and there were none in the large vessels. A few of the bodies were seen in the animals treated with pig chondroitin. In sections from both groups of animals there were metachromatic or orthochromatic granular casts in the collecting tubules (Fig. 22) as well as in an occasional dilated cystic collecting tubule. The epithelium of the collecting tubules, mainly in the inner stripe of the medulla, included scattered densely basophilic cells.

Discussion

The available evidence favors the conclusion that acid polysaccharide released from cartilage by papain, combines with basic plasma protein to form an acid circulating component. Thus the appearance of

an abnormal acid peak in the plasma electrophoretic pattern coincides with a comparable decrease in one or more basic plasma proteins. The presence of orthochromatic PAS-positive, pleomorphic material in blood vessels coincides with the occurrence of structurally similar metachromatic aggregates in lymph vessels. The blood vessel material, while staining orthochromatically in tissues prepared with neutral or weakly acid formalin fixatives, stains metachromatically in tissues prepared with Carnoy's or Bouin's solutions. This could be explained on the basis of a dissociation of the protein-polysaccharide complex under the conditions of fixation. Thus the blood material is rendered metachromatic with the Carnoy fixative which, lacking formalin, is well known to dissolve rather than to precipitate or fix basic proteins.[3] Another example of the failure of these two fixatives to demonstrate basic tissue proteins is their inability to preserve the eosinophilic granules of Paneth cells.[5] Dissociation of the polysaccharide protein complex in the Bouin fixative, with restoration of the metachromasia of blood aggregates, in all probability depends on the strong acidity of this solution. In this case the pH at which the polysaccharide-protein complex dissociates, correlates well with the pH at which metachromasia of chondroitin sulfate is suppressed; i.e., below pH 2. This suggests that combination of the acid polysaccharide with either the basic dye or basic proteins involves a similar type of linkage, possibly an ionic bond.

The disparity in the manifestations following the introduction of papain on the one hand and chondroitin sulfate on the other is surprising. In particular, the absence of blood aggregates and the alterations in the kidney in rabbits treated with chondroitin raise several questions. The difference may be due to impurities in the commercial chondroitin preparations. The possibility remains that there are differences between the mucopolysaccharides themselves, perhaps reflecting species variations or modifications resulting from the commercial preparative procedures. It is known that there are at least three types of chondroitin sulfate and that various mammalian tissues differ in composition with respect to these substances.[6]

The pathogenesis of the renal lesions is an interesting problem which may be better understood in the light of recent knowledge of the physiology of the renal circulation. Evidence from various sources[7,8] indicates that hemoconcentration increases progressively in renal medullary vessels toward the tip of the papilla. It seems reasonable that the predilection of the globular aggregates for the vessels of the papilla in the papain-treated rabbits is related to the twofold increase

in plasma osmotic pressure known to occur there.[7] The embolic occlusion of these vessels by precipitated material has been demonstrated (Fig. 13). Such embolism might be related to the development of the tubular hemorrhages. It is difficult to explain the absence of casts in the collecting tubules in sections showing massive hemorrhages in proximal tubules except on the basis of some obstruction to the progress of the blood down the tubules. The dense metachromatic casts observed in some distal tubules (Fig. 8) may be important in this respect.

Hemoconcentration may also be a factor in the deposition of PAS-positive globules in glomerular and post-glomerular capillaries after the injection of beef chondroitin. However, no plausible explanation can be suggested for the cortical distribution of this material as compared with the predominantly medullary distribution of the intravascular material after papain administration. The metachromatic casts in collecting tubules of rabbits treated with chondroitin resemble those noted by Oliver in collecting tubules of animals with proteinuria arising presumably from the formation of a protein-sulfated mucopolysaccharide complex.[9]

Summary

Histologic changes occur in the blood, kidneys and liver following the intravenous administration of papain. In blood vessels, polymorphous, PAS-positive aggregates are seen which appear orthochromatic with selected stains following formalin fixation and metachromatic after fixation with Carnoy's or strongly acid Bouin's solution. The plasma contains an abnormal acid component in the electrophoretic pattern; and the plasma smears dry with a frosty appearance. The kidneys show severe tubular hemorrhages which are more clearly demonstrated and localized by a new procedure for staining blood in tissue sections. Depletion of glycogen and basophilia in periportal liver cells persists many days after the administration of papain. Rabbits injected with chondroitin sulfate do not reveal aggregates in the blood vessels but do show renal changes which differ from those observed following the introduction of papain.

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[*Illustrations follow*]

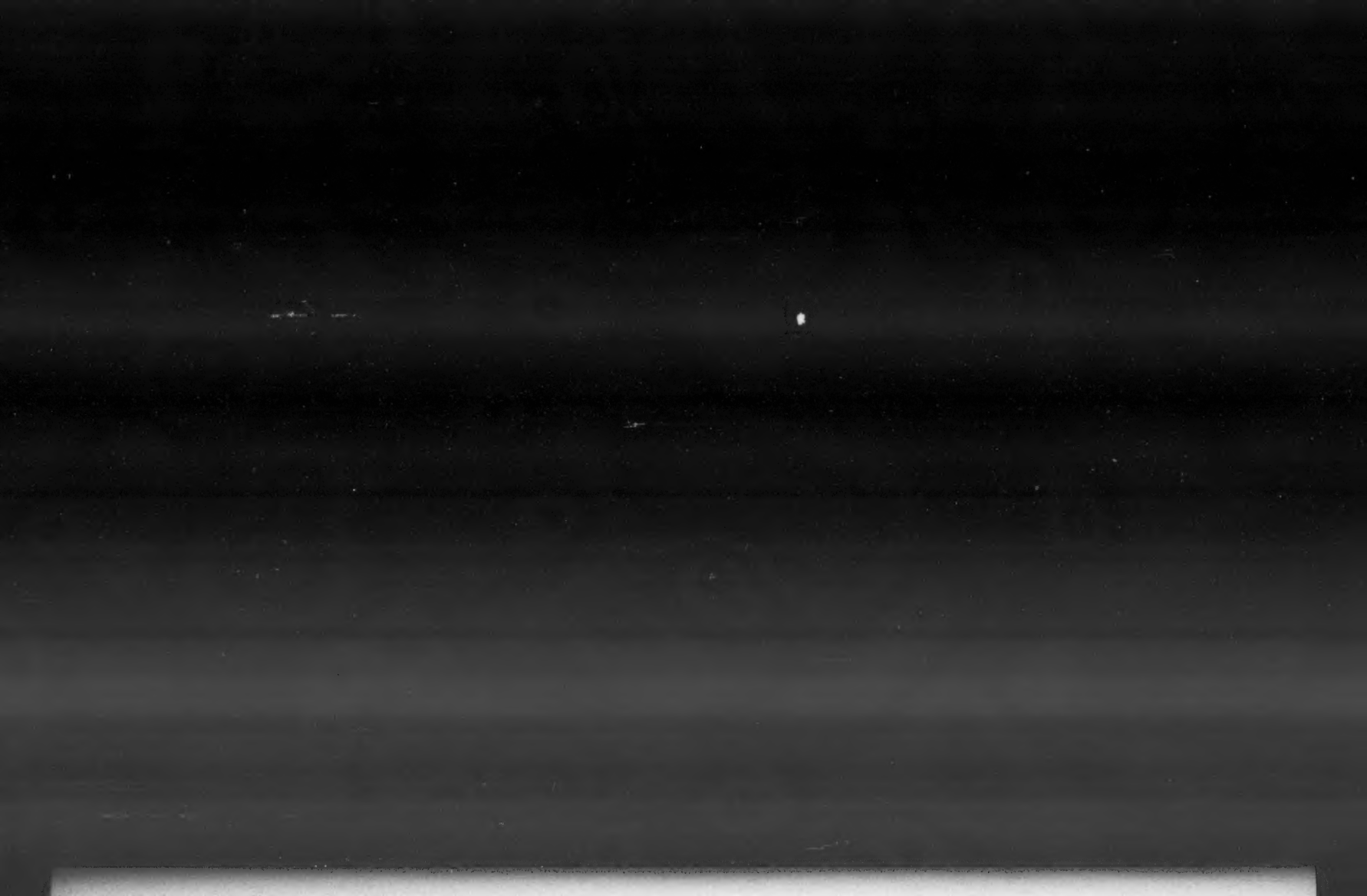
LEGENDS FOR FIGURES

FIG. 1. Orthochromatic fibrils in blood clot 1 day after papain administration. Fixed in A.A.F., embedded in paraffin. Azure A stain at pH 4.5. × 260.

FIG. 2. Metachromatic material in vessels of the liver 1 day after introduction of papain. Bouin's fixation. Azure A stain at pH 4.5. × 130.

FIGS. 3. & 4. Electrophoretic plasma pattern of rabbit 1 day after papain administration (Fig. 3) and control rabbit (Fig. 4), showing abnormal acid component and decreased basic protein in experimental animal. Oxalated plasma is diluted 3-fold with, and dialyzed 18 hrs. against 0.1 ionic pH 8.5 veronal buffer in the cold. Descending boundary recorded at 2 hours.

FIGS. 5. & 6. Phase contrast photomicrographs showing structural detail of the frosty dried plasma smear from papain treated animal (Fig. 5) and the transparent dried plasma smear of control rabbit (Fig. 6). × 515.



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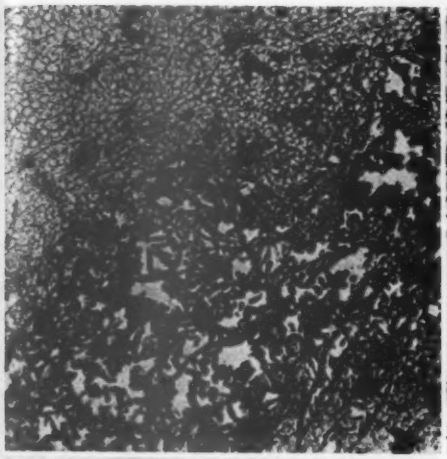

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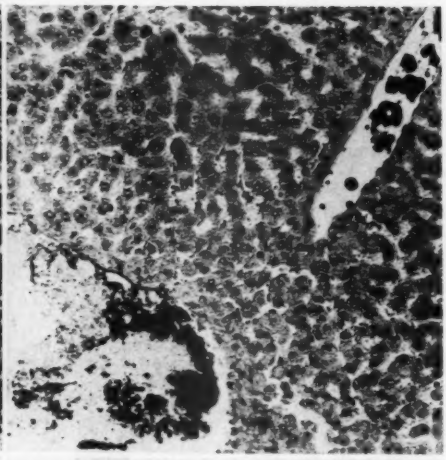

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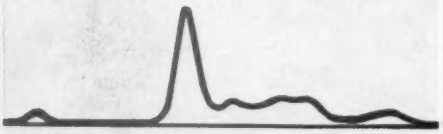

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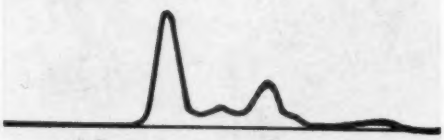

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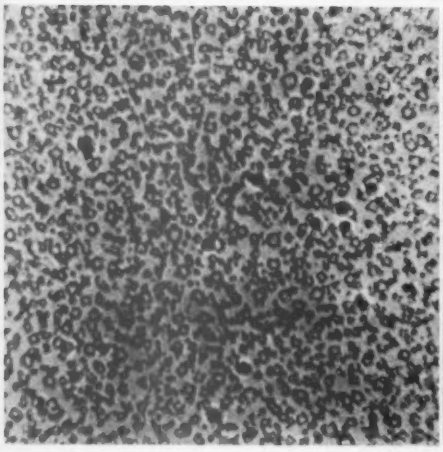

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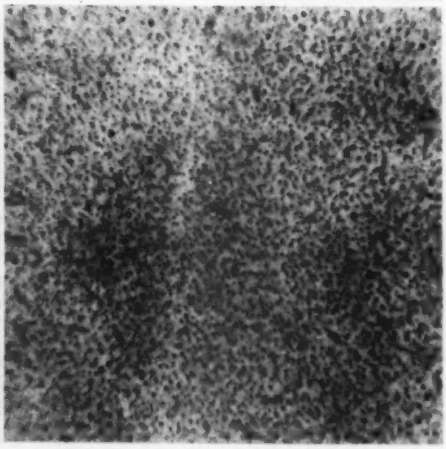

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FIG. 7. Outer renal medulla of rabbit 1 day after administration of papain, showing hemorrhage and metachromatic debris in proximal straight tubules and metachromatic material in capillaries. Bouin's fixation. Azure A stain at pH 4.8. × 205.

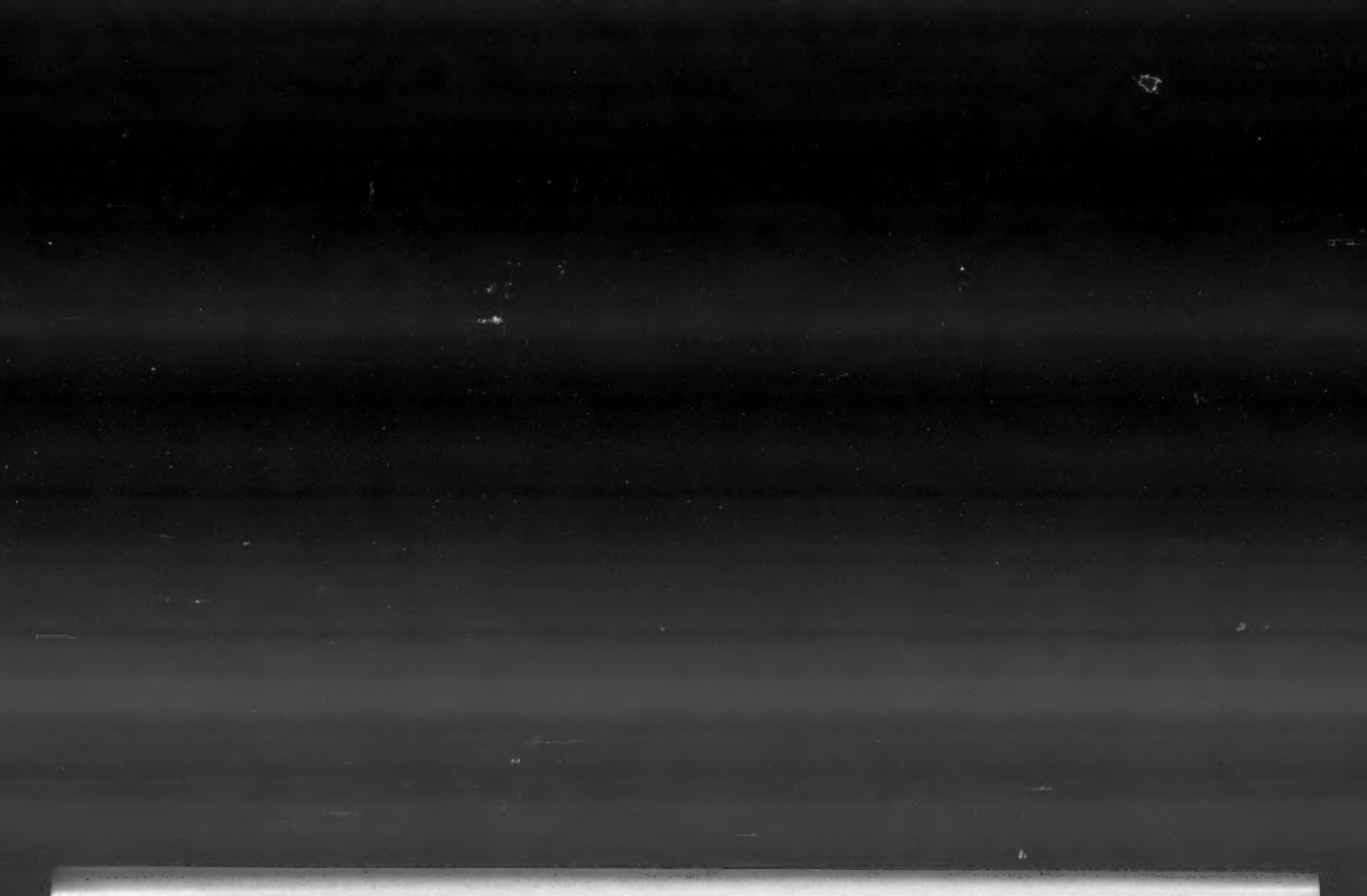
FIG. 8. Renal cortex of same rabbit, showing metachromatic cast in distal tubule. Carnoy's fixation. Azure A stain at pH 4.5. × 285.

FIG. 9. Outer renal medulla 1 day after injection of papain, showing tubule hemorrhages. Sections fixed in Bouin's fluid were rinsed 90 seconds in water and stained with azure A at pH 2.5. × 63.

FIG. 10. Renal medulla of same rabbit, showing metachromatic material in blood vessels. Bouin's fixation. Sections were rinsed thoroughly as usual prior to azure A staining at pH 4.8. × 60.

FIG. 11. Renal medulla 1 day after administration of papain, showing metachromatic globules in small vessels. Bouin's fixation. Azure A pH 4.5 stain. × 260.

FIG. 12. Renal papilla of the same animal, showing globules in vessels. PAS stain. × 230.



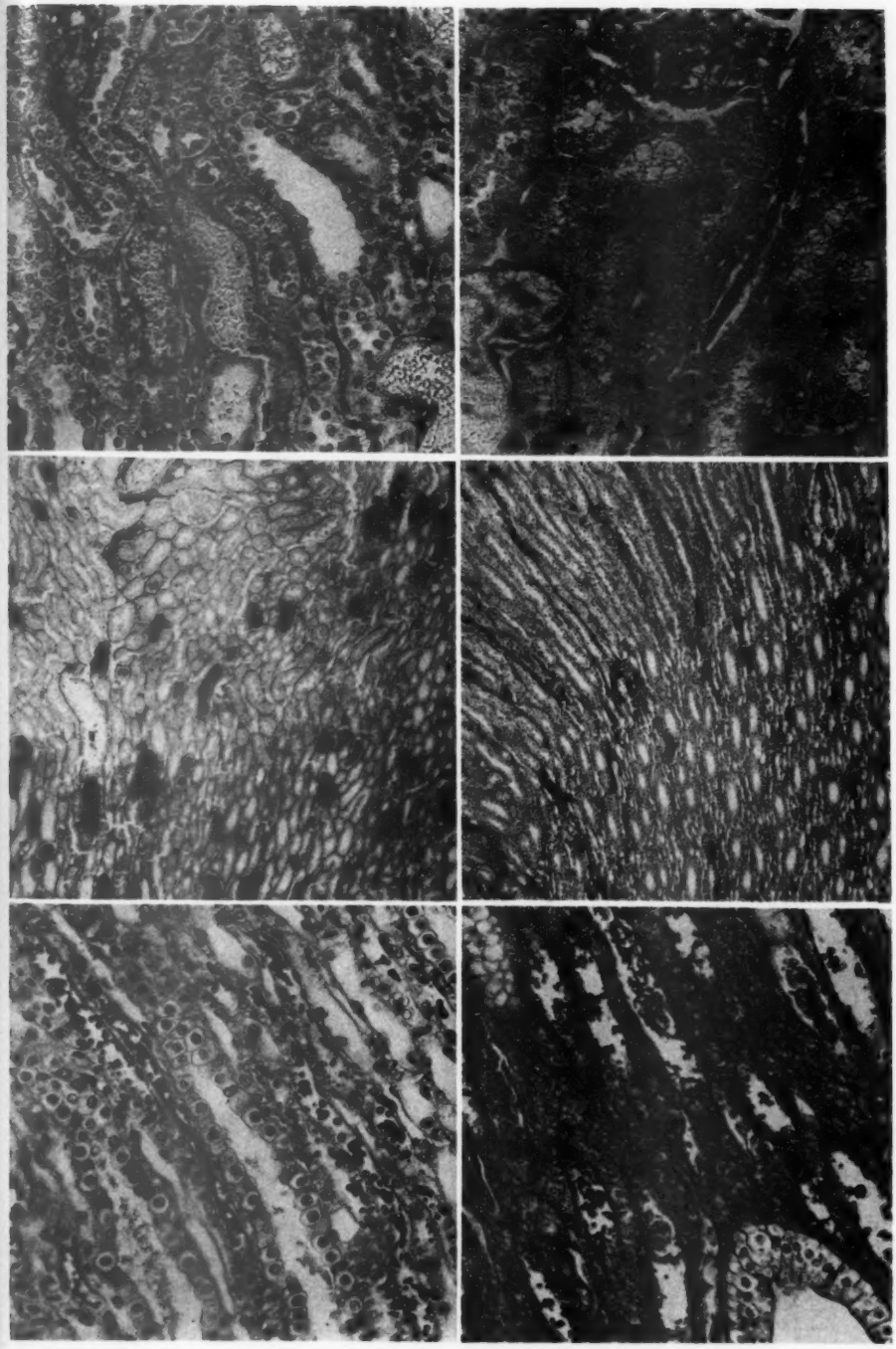


FIG. 13. Renal papilla 1 day after introduction of papain, showing embolization of small vessel by metachromatic debris. Bouin's fixation. Azure A pH 4.8 stain. × 310.

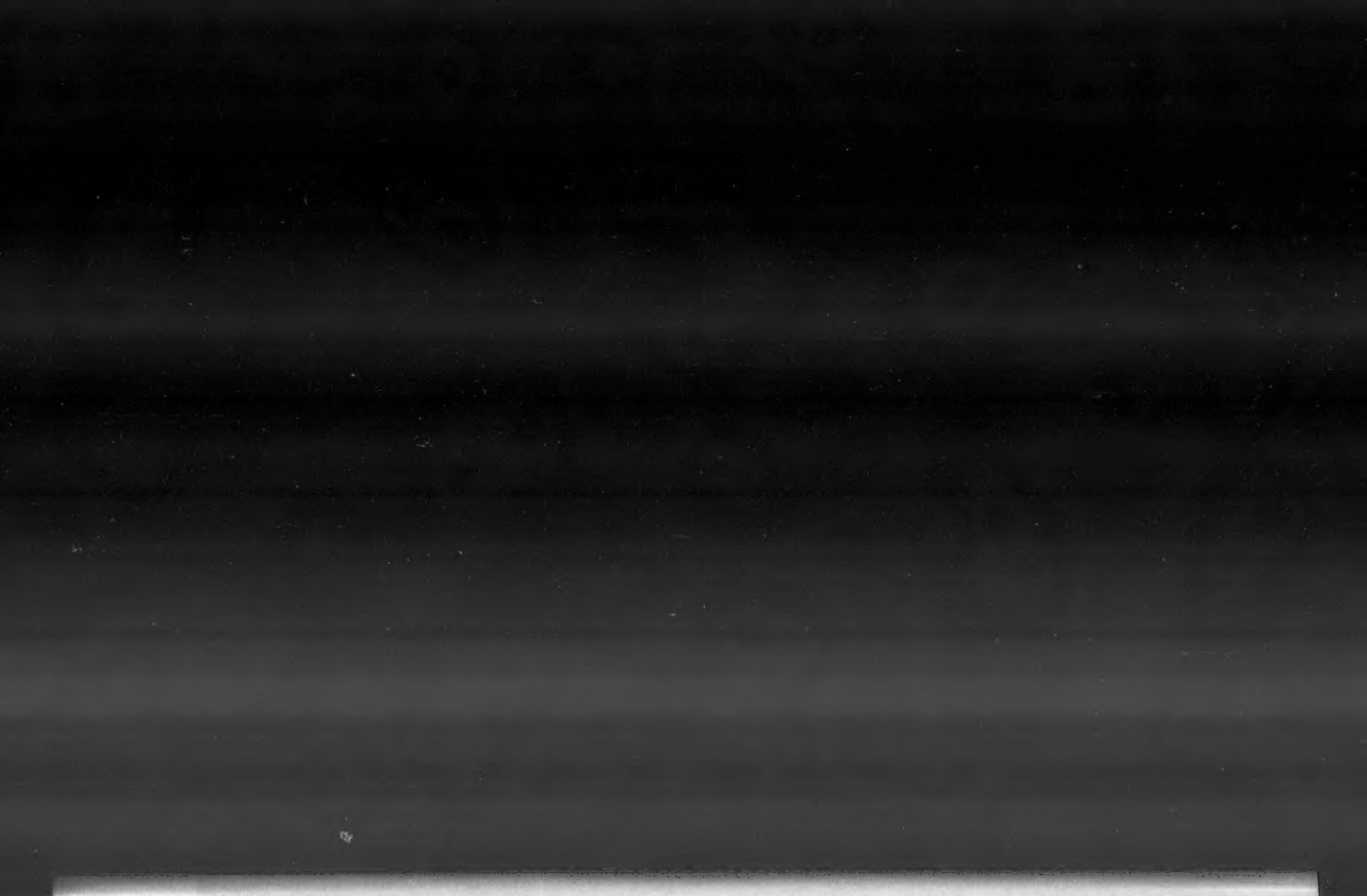
FIG. 14. Outer medulla 3 days following injection of papain, showing greenish hyalinized casts. Neutral formalin fixation. Azure A stain at pH 4.5 × 200.

FIG. 15. Renal papilla 9 days after introduction of papain showing casts in collecting tubules. PAS stain. × 105.

FIG. 16. Liver of moderately affected rabbit 1 day after papain injection. Note striking depletion of periportal glycogen. PAS stain. × 70.

FIG. 17. Liver of control rabbit, showing clear cytoplasm in periportal region. Azure A pH 4.5 stain. × 260.

FIG. 18. Liver of rabbit 22 days after papain administration, showing persistent basophilia. Azure A pH 4.5 stain. × 260.



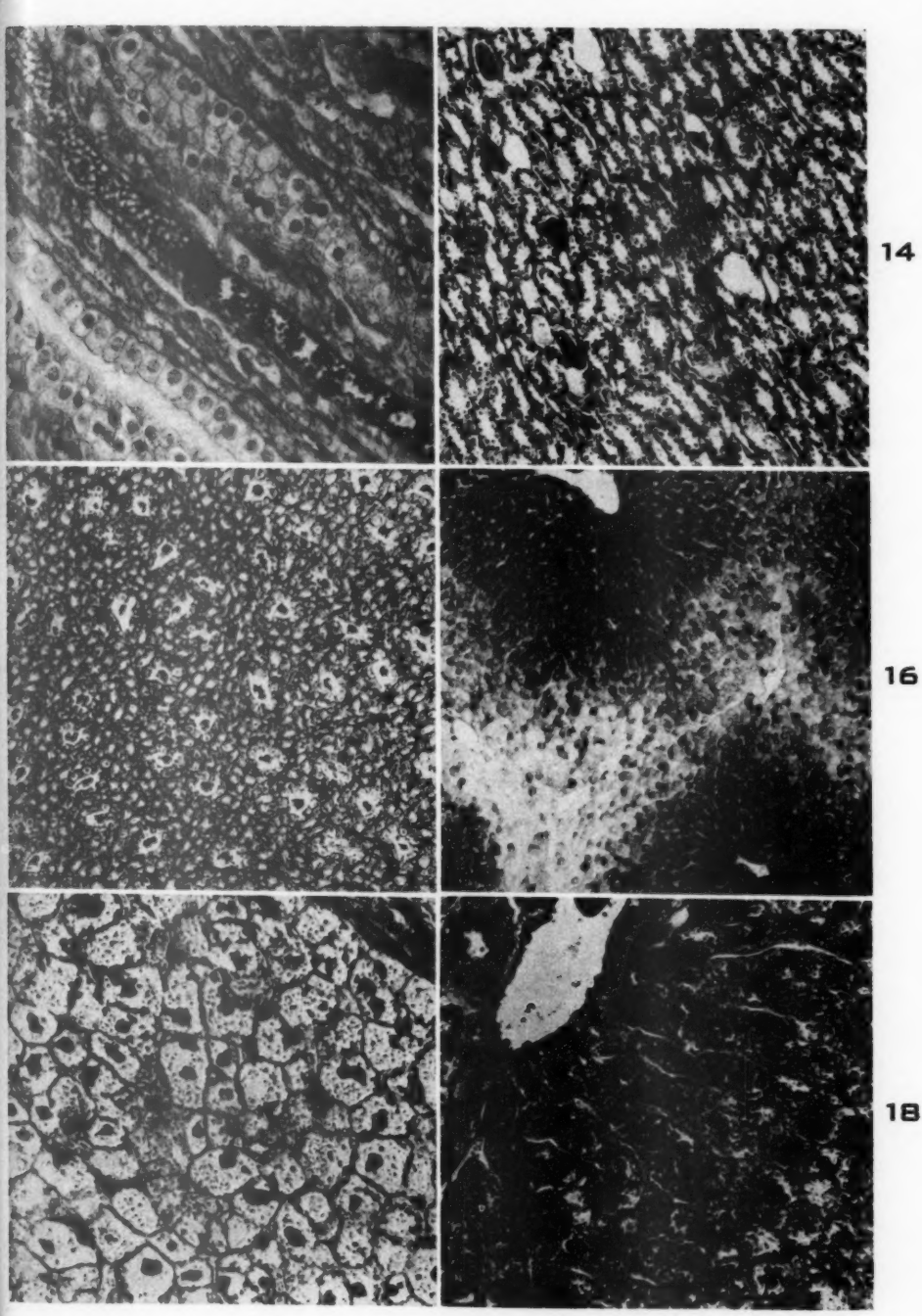

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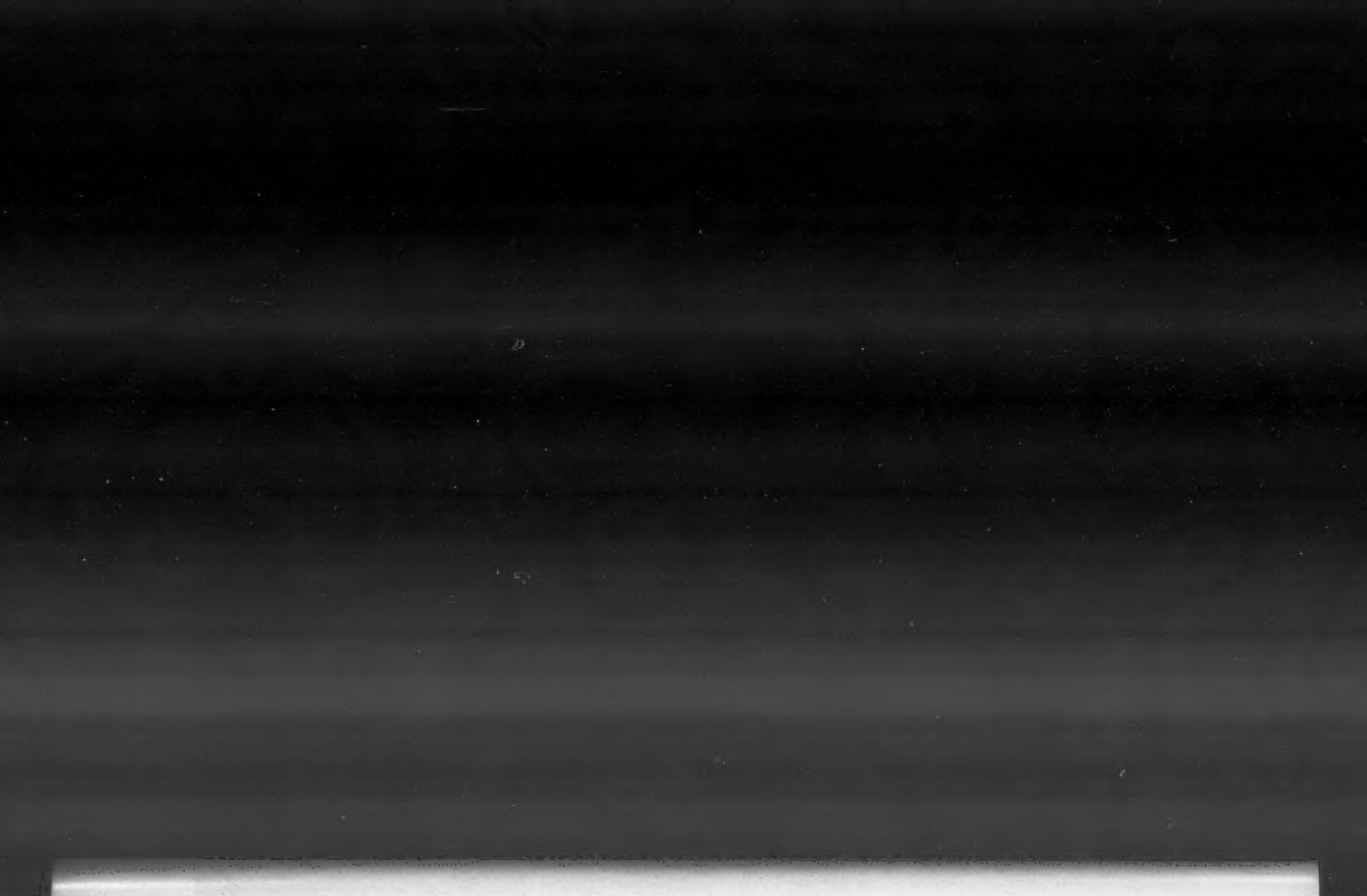
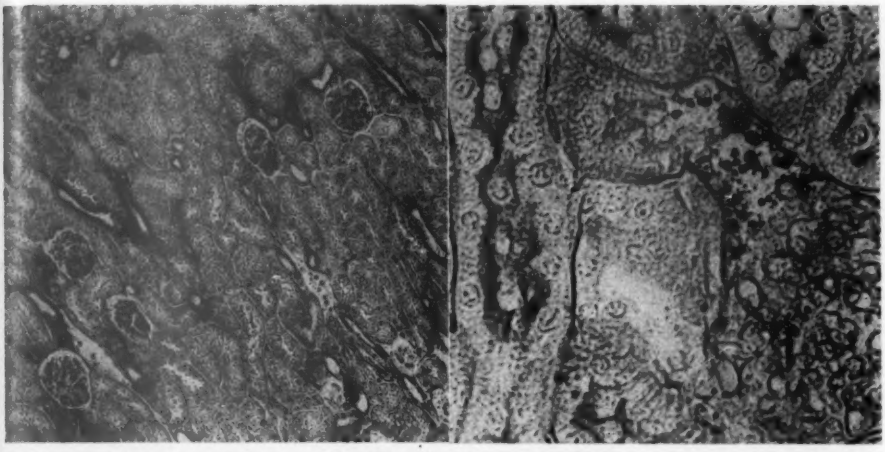
FIG. 19. Kidney 4 hours after injection of 3 gm. of pig cartilage chondroitin sulfate. Note metachromatic epithelium of distal tubules. A.A.F. fixation. Azure A pH 4.5 stain. × 100.

FIGS. 20 and 21. Kidney 5 hours after injection of beef cartilage chondroitin sulfate, showing dark red bodies at the origin of proximal convoluted tubules and in glomerular and postglomerular capillaries. Bouin's fixation, PAS stain. × 420.

FIG. 22. Renal papilla 4 hours after injection of pig cartilage chondroitin sulfate, showing metachromatic granules in collecting tubules. A.A.F. fixation. Azure A pH 4.5 stain. × 260.





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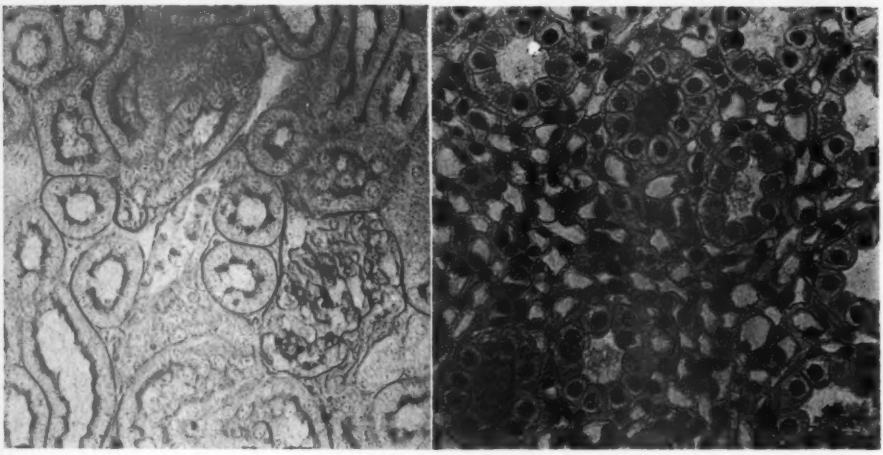

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MALFORMATIONS CAUSED BY NECROSIS IN THE EMBRYO*

ILLUSTRATED BY THE EFFECT OF SELENIUM COMPOUNDS ON CHICK EMBRYOS

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In the early days of scientific teratology many of the concepts concerning the development of malformations were speculative. It was assumed that development of a primordium must have stopped or deviated, or that it must have split, fused, or failed to do so. With adequate knowledge of normal development it should be possible to determine the stage at or before which the abnormality occurred. Only in recent years has information accumulated to indicate that pathologic regression of previously well formed parts is an important process in the production of embryonic malformation. These processes can cripple or destroy a primordium long after it has formed normally, and this can only be recognized when it is actually observed in appropriate developmental stages of abnormal embryos.

Selenium-induced malformations in chick embryos represent excellent examples of this mechanism, and are therefore of basic interest. A description of findings in embryos affected by selenium will be followed by a discussion of the significance of the destruction of tissue in the pathogenesis of malformations.

Ever since the investigation of malformations occurring in chicken eggs in certain areas of the Middle West[1,2] the teratogenic effect of selenium compounds has been well known. Malformed embryos were found in eggs laid by hens which had consumed selenium-containing foods, and similar abnormalities were produced by the injection of selenium compounds into eggs.[3] There appeared, among others, defects of the brain, eyes, beak, and extremities.

Since the sequence of events in these malformations had never been investigated, a study of embryos from the eggs laid by selenium-fed hens was undertaken.† Hens were fed a ration containing 45 per cent wheat to which selenium had been added (17 parts per million). This resulted in the ingestion of 8 parts of selenium per million in the total ration. Eggs were gathered beginning 2 weeks after the inception of the diet. Of 245 eggs thus procured, 214 were fertile and 2 of these

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† Eggs were procured through the cooperation of Drs. W. Kohlmeyer, G. S. Hershfield, and A. B. Hoerlein of the South Dakota State College of Agriculture and Mechanic Arts. Dr. A. E. Scheinermann assisted in the examination of the chick embryos.

contained dead embryos when opened. The embryos for the present study were obtained from eggs incubated for 1½, 2, 2½, 3, 4, and 5 days. They showed no conspicuous abnormalities to gross examination. Fixation was accomplished in Bouin's solution, and specimens were shipped in 70 per cent alcohol. Serial sections were prepared of 2 embryos of 1½ days, 2 of 2 days, 1 of 2½ days, 5 of 3 days, 5 of 4 days, and 7 of 5 days of incubation.

For comparison, several serially sectioned normal chick embryos were used. Two of these proved to be particularly suitable. The younger one with 30 pairs of somites (approximately 2½ days of incubation) represented the stage at which lesions first appeared. The older one, of 4 days and 5 hours incubation, was comparable with the 5-day experimental embryos since the latter were retarded in development. Control studies of eggs of untreated hens were not specifically carried out to rule out the effects of hereditary or undesirable environmental factors. These have not appeared important in the large volume of incubations observed in the same institution through the years. There is no doubt that virus infection may produce necrosis of embryonic tissues. With infection by Newcastle disease virus[4] the distribution of lesions is determined by the route of inoculation. Although it is conceivable that infection acquired in this manner might produce a distribution similar to that reported here, the absence of abnormalities of this nature in embryos of untreated hens, and the reproduction of late stages of the malformations by direct injection of selenium compounds into eggs[3] establish with a reasonable degree of certainty that the changes described are in this instance the effects of selenium.

Observations

Three types of abnormalities were observed in the embryos. The most obvious, which could be recognized and evaluated without difficulty, was characterized by necrosis evidenced by pyknosis and fragmentation of nuclei, phagocytosis, disintegration of tissue, and occasionally hemorrhage into affected areas. The recognition of focal developmental retardation, resulting in abnormal size, shape, or topographic relations of parts of organs was more difficult. The third type of abnormality was characterized by a general retardation in the development of the entire embryo.

Table I lists the abnormalities (except general retardation) found in the present material. None of the embryos of 1½ and 2 days, and only one of the 3-day embryos showed discernible abnormalities. Another of the 3-day embryos was very severely malformed in a manner

which differed from the others. This was presumably unrelated to the effect of selenium. The remaining embryos exhibited significant alterations.

The only 2½-day embryo sectioned showed a few fragmented nuclei in the diencephalon, rhombencephalon, and the spinal cord. Nuclear fragments and a few macrophages were present in the optic cups and lens vesicles. The telencephalon contained disintegrated nuclei in its rostral portion. The caudal end of the embryo had been torn off in the process of preparation.

Among the 3 embryos of 3-day incubation to be described regressive nuclear changes appeared in the spinal cords of 2 and in the caudalmost somites of one. Embryo # 5 in this group had conspicuous lesions which justify detailed description (Figs. 1-6). The telencephalon was rudimentary, showed no hemispheres, and its wall contained many necrotic cells. The nasal pits were shallow and near each other, medial to the eyes. Figure 1 shows the peripheral portion of one of the nasal pits. The very shallow center is about 40 μ distant. The diencephalon had extensive destruction in those portions of its wall which, as shown in older embryos, were characteristically damaged. The areas affected included the floor and the ventral portions of the lateral walls near the optic stalks (Figs. 1 and 2). Pyknotic and fragmented nuclei were numerous in these portions, and the ventricle contained necrotic cells and macrophages. Similar changes were seen in the optic cups (Figs. 1 and 2), which were also abnormal in shape. The two layers were separated by a wide ventricle, which contained debris as did the third ventricle of the brain. The lens showed similar but less necrosis and debris in the lumen. In the lateral wall of the rhombencephalon there were fragmented nuclei rostral to the optic vesicles (Fig. 3), but there was no disruption as in the diencephalon.

The spinal cord was the most consistently affected part of the embryo. The cranial portions were altered much as in the case of the rhombencephalon (Fig. 4). The severest change was found in the lateral walls, at approximately equal distances from the ventral and dorsal aspects. The caudal portions, beginning at a level somewhat cranial to the posterior intestinal portal, showed more extensive and irregular destruction, with focal disruption of the architecture. At these levels the dorso-medial portions of the somites also showed nuclear changes indicative of necrosis (Fig. 5).

The tail was in the stage at which primordia ordinarily differentiate from the trunk-tail-node. It appeared that the dorsal portion in which the spinal cord formed was defective, and a very small group of cells

TABLE I

List of Abnormalities Found in Serially Sectioned Chick

Age—days	1½		2		2½	3				
No.	1	2	1	2	1	1§	4	2	3	5
Telencephalon N*	−	−	−	−	+		−	−	−	++
Telencephalon R†	−	−	−	−	−		−	−	−	++
Diencephalon N	−	−	−	−	+		−	−	−	++
Mesencephalon N	−	−	−	−	−		−	−	−	−
Rhombencephalon N	−	−	−	−	+		−	−	−	+
Rhombencephalon H‡	−	−	−	−	−		−	−	−	−
Spinal cord N	−	−	−	−	+		−	+	+	++
Spinal cord R	−	−	−	−	−		−	−	−	+
Optic cup N	−	−	−	−	+		−	−	−	+++
Optic cup R	−	−	−	−	−		−	−	−	++
Lens N	−	−	−	−	−		−	−	−	+
Lens R	−	−	−	−	−		−	−	−	+
Nasal Pits R	−	−	−	−	−		−	−	−	++
Wings N	−	−	−	−	−		−	−	−	+
Legs N	−	−	−	−	−		−	−	−	−
Somites N	−	−	−	−	−		−	−	+	++
Tail N	−	−	−	−	?		−	−	−	++
Tail R	−	−	−	−	?		−	−	−	−
					Caudal end missing					Figs. 1–6

* N, necrosis.
† R, reduction in size.
‡ H, hemorrhage.
§ Severely malformed.

attached to the epidermis represented the greatly reduced spinal cord substance (Fig. 6). Both the spinal cord tissue and the somites which were beginning to differentiate, showed necrosis. On the ventral aspect, where trunk-tail-node tissue is continuous with the epidermis in an extension of the cloacal membrane, necrosis normally occurs; in the present embryo the necrosis was perhaps unusually severe. The wing buds showed a few fragmented nuclei and very few macrophages just under the epidermis; the latter was unaffected. The leg buds were normal.

In comparison, sections of a normal embryo of approximately 2½

Embryos, Derived from Hens Fed Selenium-containing Grain

4					5						
4	1	2	5	3	1	6	2	7	3	4	5
++	++	++	++	++	+	+	++	+++	+++	+++	++
+	+	++	++	++	+	+	++	++	+++	+++	+++
+	+	+	++	+	−	+	+++	+++	++	++	++
−	−	−	−	−	−	−	+	+	−	−	+
+	+	++	++	++	+	+	++	++	+++	+++	++
−	−	+	−	+	+	−	−	+	+	−	−
++	++	+++	++	++	++	++	++	++	++	+++	+++
−	−	+	+	+	+	+	+	++	++	+++	+++
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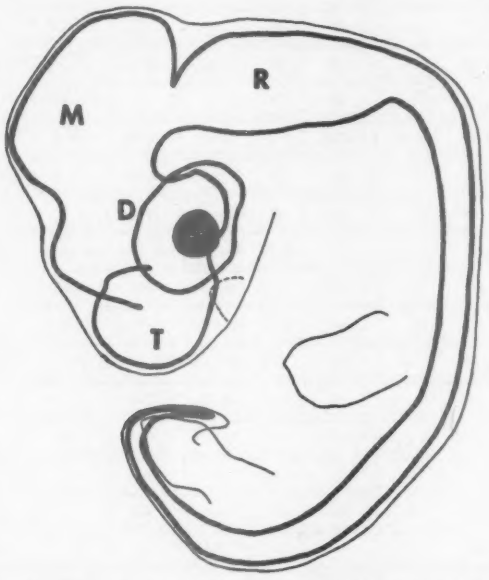
+	++	++	+++	+	+	++	++	++	++	+	++
+	+	+	+	++	+	+	?	+	+	+	++
−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−
+	−	+	+	+	+	+	+	+	++	++	+++
		Text-fig. 2 (reconstr.); Figs. 13–16	Fig. 12	Figs. 21, 22			Fig. 18		Figs. 10 11, 19, 20	Fig. 17	Text-fig. 3 (reconstr.)

days of incubation showed the following: the nasal pits were longer and deeper and were lateral to the telencephalon and rostral to the eyes (Fig. 7). The optic cups showed only minute cleftlike remnants of the optic ventricles (Fig. 8). The caudal spinal cord was well developed with a distinct central canal beyond the level at which somites had differentiated (Fig. 9). In the abnormal embryo the caudal somites had only a small vestige of spinal cord between them. In general development, the 3-day embryo of the experimental series was slightly more advanced than the 2½-day normal embryo. It could therefore be considered to be nearly normal in this respect.

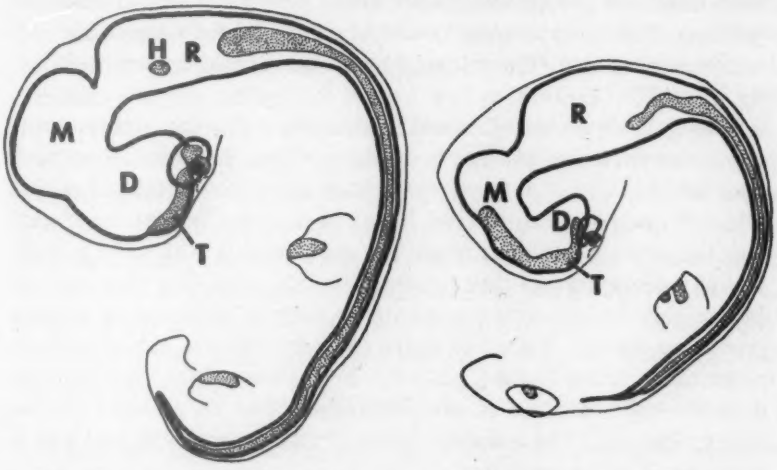
All 4-day and 5-day embryos showed distinct and severe lesions which varied only in degree. As examples, a 4-day embryo with moderately severe changes (# 2) and the most severely affected 5-day embryo (# 5) will be described in detail. Graphic reconstructions of lateral views were prepared from transverse or frontal sections of the two embryos, and from the control embryo of 4 days and 5 hours (Text-figs. 1 to 3).



Text-figure 1. Normal embryo of 4 days and 5 hours incubation, for comparison. Reconstruction of chick embryos prepared from transverse or frontal sections magnified 28 times. Various structures appear projected on a sagittal plane. Thin line: contour of body and extremities; heavy line: central nervous system and optic cup; broken line: nasal pit; solid black: lens (except areas of necrosis). Stippled areas: necrosis. Stippled and marked H: hemorrhage. Of bilateral structures, only those on the left are shown.

The most constant location of necrosis was in the central nervous system. The ventral and adjacent lateral parts of the diencephalon near the optic stalks were regularly affected (Text-figs. 2 and 3). The lesions extended rostrad onto the telencephalon to a varying extent (T in Text-figs. 2 and 3; Fig. 13), and the formation of the hemispheres was suppressed in proportion to the severity of this change. Table I shows that the shape of the hemispheres was only mildly affected in 2 of the 4-day and 2 of the 5-day embryos, and severely altered in all others. Only traces of paired hemispheres were present in one of the reconstructed specimens (Text-fig. 2); the other one was completely

devoid of them. In the latter instance the areas of necrosis extended over the rostral end of the brain to the dorsal aspect, and could be followed into part of the mesencephalon (Text-fig. 3, D and M). Two additional 5-day embryos showed abnormalities of the mesencephalon (# 2 and # 7). The caudal portion of the mesencephalon and the rostral part of the rhombencephalon were generally unaffected except that hemorrhage occurred in 5 of the embryos at or near the point of emergence of the fifth cranial nerve (Text-fig. 2, R, and Fig. 10). The hemorrhages were not associated with discernible necrosis, and their cause was unexplained. A constant area of necrosis was found on either side of the rhombencephalon beginning caudal to the otic vesicles and ventral to the thin portion of the roof (Text-figs. 2 and 3, and Fig. 14). This continued into the lateral walls



Text-figure 2. 4-day embryo (# 2).
Text-figure 3. 5-day embryo (# 5) showing selenium-induced damage.
D, diencephalon; M, mesencephalon; R, rhombencephalon; T, telencephalon.

of the spinal cord in every case (Fig. 15). The appearance of this lesion was fairly constant down to a level somewhat caudal to the wing buds; from there on the severity varied and in some instances the spinal cord was greatly reduced in width and its dorsal portion was occasionally absent (Text-fig. 2 and Fig. 19). Only in one instance (Text-fig. 3 and Fig. 14) did a continuous and identifiable spinal cord terminate about the level of the leg buds. The spinal ganglia were sometimes well developed in areas in which the neural tube was severely reduced (Fig. 17).

The distal part of the spinal cord and the structure of the tail deserve

special consideration. At 2½ days of incubation, just before the time when lesions usually became apparent in the experimental chicks, normal embryos have a well differentiated neural tube beyond the level of the leg buds, and reaching the base of the tail (Fig. 9). Caudally, the tube merges with the undifferentiated tail bud. Differentiation of somites and notochord lags behind that of the neural tube so that the caudal part of the neural tube is not accompanied by either of these structures in cross sections of the tail, but rather by undifferentiated mesenchyme from which they are to develop. As described previously, 4-day and 5-day embryos showing the effects of selenium, had a greatly reduced spinal cord in the caudal part of the trunk. Only small cell groups were encountered in the tail in a few instances (Fig. 12) and traces of necrotic cells were occasionally seen. In other cases there were small cell groups which were either vestiges of neural tissue, or portions of somites in areas in which a well defined neural tube was completely missing. The tail might also be reduced in length (Text-fig. 3).

The optic cups were abnormal in all 4-day and 5-day embryos, and the lenses were also affected in most instances. Evidence of necrosis was found in the cups in every instance, and was associated with a widened space between the two layers of the cup. In some specimens cup formation was almost abolished and a vesicle with only a slight lateral indentation was then present (Fig. 18). All eyes were reduced in size, and the reduction was greater in those in which necrosis was a prominent feature. Only two 4-day embryos failed to exhibit definite abnormality of the lenses (Table I). In all others there were necrosis, defective differentiation of lens fibers, and often an enlarged central cavity (Fig. 18). The reduction in the size of the optic cup and lens is evident in Text-figures 1 to 3.

The nasal pits showed no evidence of necrosis. However, they were reduced in size and the epithelium was not as tall as in normal embryos. More conspicuous was the abnormal position of the nasal pits which were found more ventrally and medially than normal. The pits were medial to the eyes and near each other (Fig. 13). Maximal changes in size and position were found to be associated with severe defects of the cerebral hemispheres.

The wing and leg buds showed necrosis in all 4-day and 5-day embryos; however, the severity varied greatly. A constant occurrence in the areas of necrosis was the appearance of clear spaces under the epidermis containing scattered macrophages and engulfed cellular debris. These spaces, which are never seen in normal embryos, were often easier to spot than the few fragmented nuclei of mesenchymal

cells in their vicinity. The overlying epidermis showed no abnormalities. The severity of necrosis is exemplified by Figures 19 to 22 which exhibit respectively, a moderate change, and the severest alterations encountered in this study. In most instances only scattered cells were affected, and examination of the sections revealed no abnormality in the shape of the buds. Only in specimens with severe damage was there gross deformity, such as the thin bud shown in Figure 21 or the undersized limb buds in Text-figure 3.

Since none of the embryos had been incubated longer than 5 days, it was impossible to determine how much longer necrosis continues to be manifest in embryos of selenium-fed hens, and in what way the final form of the defects develops once the acute phase of cell destruction has ended. However, the early defects leading to abnormalities of the nervous system, eyes, beak including the nose, and extremities, have been demonstrated. In order to obtain later stages, direct injections of selenium compounds into eggs prior to incubation was attempted according to the method of Franke and co-workers.[3] This did not have results as consistent as those obtained with the feeding of hens. This was also Landauer's[5] experience. A few older embryos so treated showed deformities of the third and lateral ventricles of the brain (Fig. 23) as well as reduction in the size of the hemispheres (Figs. 23 and 24), microphthalmia (Fig. 24), and abnormalities of the spinal cord. These are presumably later stages of the lesions described above. None of the lesions were found consistently in any of the 5 serially sectioned 2-week embryos. There was no evidence of recent necrosis in these specimens.

Discussion

As far as has been ascertained, the visible primary effect of selenium compounds transmitted to the egg by the hen, was necrosis of cells in established portions of the embryo. This occurred regularly in certain well defined areas of the brain and spinal cord; in the optic cups and lens vesicles, in the mesenchyme of the limb buds and, to a slight degree, in somites of the tail region. Some of the malformations encountered by earlier investigators in older embryos, could be traced to defects resulting from the areas of necrosis. These included microcephaly, microphthalmia, and anomalies of the extremities.

Added to these defects were others which appeared in parts not known to have been the seat of necrosis. In these instances necrosis occurred in nearby tissues which are in some manner correlated in their development with the parts under consideration. This was exemplified by defects of the upper beak and nearby portions of the face, features

resembling minor degrees of cyclopia. The early stages noted in the foregoing account consisted of a reduction in the size of the nasal pits, and a variation in their relation to each other and to the midline. This change was associated with, and perhaps caused by, severe defects or complete absence of the telencephalon in turn attributable to necrosis. Assuming that this was true, one may postulate that the defects of the telencephalon were followed by a failure of development of median portions of the face, even though their substance was not intrinsically damaged. In other words, the normal developmental pattern of the face appears to depend on the presence of a normal telencephalon, and is altered when the latter is defective. It will be recalled that in cyclopia a somewhat similar mechanism has been demonstrated: the neural plate may not be defective primarily, but may be influenced by a reduced mesodermal substratum.[6-8] In the present cases, microphthalmia may have contributed to the further reduction of the size of the face.

Similar considerations may possibly apply to the caudal portion of the body. Interpretation is rendered more difficult by our lack of knowledge concerning the developmental mechanism of the tail bud. It has been observed that the severity of the defects in the spinal cord caused by necrosis, increases in a caudal direction. One may be tempted to attribute complete absence of the spinal cord in the tail to very extensive necrosis. Against this assumption is the small size of the neural primordium in the early stages of its differentiation from the trunk-tail-node and the absence of evidence of necrosis of sufficient extent to account for the reduction in size. One might suspect that differentiation of the neural tube in the tail bud depended on continuity of its precursor substance with a more or less intact neural tube. Thus, in the absence of continuity, the neural tube would fail to differentiate to the usual extent. There is no information at hand concerning the mechanism of differentiation of the tail bud, which might substantiate or refute this concept. The fact remains that here again there was a "median defect" with approximation of bilateral primordia (Fig. 11).

In the eyes, secondary changes may have obscured the course of development of malformations caused by selenium compounds. The presence of an optic vesicle rather than a cup (Figs. 1, 2 and 18) does not indicate the end of normal development at the vesicle stage. At 2½ days of incubation when most of the experimental embryos showed no morphologic abnormalities, the optic cups were fully formed and only a faint trace of the optic ventricles remained (Fig. 8). It must be assumed, therefore, that the presence of a cavity between the layers of the cup was a secondary change, caused in some manner by necrosis

and possibly by the accumulation of an exudate with a differential retardation in the growth of various parts of the cup. Similarly, the form of a vesicle might reappear in the lens as the result of extensive degenerative changes (Figs. 2 and 18).

There is no information concerning the extent to which secondary processes such as regeneration, healing of defects, or developmental interrelationship of parts occur and modify the defects described here after the fifth day of incubation. The role of these processes in x-ray induced malformation has been emphasized recently by Hicks.[9]

Focal retardation of development has been described at several points in this report. General retardation also occurred: both are demonstrated by a comparison of Text-figures 2 and 3 with the normal embryo shown in Text-figure 1.

TABLE II

Measurements in Microns of Malformed Embryos and a Normal Control, Taken from Reconstructions (Text-figs. 1 to 3)

	Normal	Malformed, average	Malformed, severe
Age	4 days 5 hours	4 days	5 days
Length	7500	6130	4630
Diencephalon	2000x1750	1630x1130	1250x750
Optic cup	1800x1320	700x500	450x300
Lens	650x580	250x250	200x80
Spinal cord			
at wings	430	200	130
at legs	400	180	0

It is tempting to speculate on the manner in which selenium compounds affect embryonic development, but the amount of information available is insufficient. Landauer[10] has quoted investigations indicating that glutathione or sodium monosulfide, as well as arsenic compounds, counteract the teratogenic effect of selenium to some extent, but these observations have not elucidated the mode of action. Moreover, it is not known when selenium, deposited in the egg before it is laid, first reaches the embryo, and how it is distributed there. It may be that the characteristic localization of necrotic lesions is the result of the distribution of the agent, or to a differential susceptibility of various tissues.

The Role of Necrosis in Abnormal Development

The present observations furnish excellent examples of malformations caused by degenerative changes in previously well formed parts. Similar abnormalities are known to occur spontaneously and as a result

of hereditary or experimentally induced defects of many different kinds. The significance of degenerative change as one of the principal teratogenic mechanisms has been appreciated only recently. The occurrence of necrosis as a normal factor in the development of the embryo is often ignored in the teaching of embryology; it is nevertheless a well known phenomenon. In fact, those who have searched for evidence of it in normal embryos, have encountered many examples.[11,12] In the brains of embryo rabbits, for instance, degeneration may be nearly as severe as that encountered in the present group of chick embryos.[13] In some instances, one is tempted to suspect that the necrosis is merely an exaggeration of the normal mechanism.[14]

A review of the voluminous literature dealing with malformations of the embryo produced by necrosis is beyond the scope of this paper. A few examples may suffice. As far as can be judged by the illustrations, the effects of radioactive phosphorus on chick embryos[15] closely resemble the lesions in the nervous system and limb buds induced by selenium. Necrosis in the neural tube has been produced by nitrogen mustard in the amphibian, Triturus[16]; by urethane in mice[17]; and by a variety of agents including x-rays in mice and rats.[18,19] Malformations of the eyes accompany alterations in the nervous system induced by radiation and nitrogen mustard. Necrosis resulting in defects of the extremities, usually apparent in later stages than described here, occurs under a variety of circumstances; e.g., hereditary abnormalities in the rabbit,[20,21] the administration of nitrogen mustard to amphibians[16]; following the administration of vasopressor substances to rat fetuses[22,23]; vitamin F (linoleic acid) deficiency in the rat[24]; and oxygen deficiency in the chick.[25]

A variety of other malformations, many of them genetic in origin, are known to result from necrosis during embryonic life. The time at which the degeneration occurs, while highly constant for each of the genetic abnormalities, ranges from the early embryo to the neonatal period. These changes do not differ basically from heredo-degenerative disease of the adult. It is interesting to note that in the mouse the condition known as "rodless retina" develops as a hereditary trait during the early postnatal period as the result of an arrest of development, whereas in the rat a similar trait follows degeneration of a normally formed retina somewhat later in postnatal life.[26] The tail is subject to many forms of degeneration in the development of mammals and birds. Abnormal reduction in size or absence of the tail caused by genetic or environmental factors, have been studied in considerable detail in several species. Some of the degenerative lesions encountered in the

limbs are associated with similar alterations in the tail. These have been produced in the rat by vasopressors[22,23] and by the use of insulin in chick embryos.[27,28] Several hereditary traits of mice and chicks reveal a timing of necrosis which is constant for each trait, and different from that of others.[14,29]

Defects of the skin and soft tissues, formerly ascribed to the effects of amniotic adhesions, are now attributed to primary intra-uterine disease leading to tissue destruction. Amniotic adhesions, when present, are presumably secondary to the uterine disorder.[30,31] In cattle there is a hereditary trait in which skin defects of the extremities, combined with abnormalities of the leg, ear, muzzle, tongue, and other parts occur in a ratio of one abnormal to three normal offspring.[32]

Atresia of the digestive canal is the result of secondary changes in a tube with a previously patent lumen. The canal is formed as the embryo elevates itself above the level of the germinal disc and acquires its body form. When an embryonic body is formed, a continuous digestive canal must of necessity be present as well. Interruptions of the lumen of the alimentary tract must therefore be secondary. That they can develop late in embryonic life, is shown by the presence of cornified epithelium beyond the point of atresia.[33] In atresia of the larger bile ducts a similar situation exists. The ducts must have been continuous in the early embryo since their branches give rise to the epithelial primordia of the liver parenchyma.

The brain of the human fetus may be the seat of focal or diffuse degeneration, at stages much older than those in the present study. In newborn infants one may find rarefaction of neural tissue with the formation of pseudocysts or the transformation of the hemispheres into sacs with transparent walls.[34] In experiments this may be mimicked by the effects on the brain of chick embryos of the administration of lead salts as late as the 15th day of incubation.[35] These examples suffice to indicate the prominent role of disintegration of preformed tissue in the pathogenesis of congenital malformations.

Evaluation of Concepts Relating to Abnormal Development

The timing of teratogenic processes has occupied investigators since the early days of investigative teratology. It was indicated in the introduction that the earlier concepts based on the presumption that timing was a salient factor in the production of abnormal development, require re-evaluation in the light of newer knowledge. We know that environmental and genetic factors may cause similar malformations, perhaps by similar pathogenetic mechanisms. Structural abnormalities detectable by available means may result from noxious environmental

agents and may be manifest promptly or after a period of delay. In the case of hereditary traits the change is determined at the time of fertilization; it may become manifest at a definite time, ranging from early embryonic life to old age. It is therefore clear that the time at which development begins to deviate visibly from normal is the latest time that the given abnormality could have been provoked, although provocation may have occurred at any time previously.

The pattern of susceptibility to teratogenic agents existing in an embryo at any given moment constitutes another aspect of the significance of timing. It has been amply demonstrated that rapidly growing primordia are more susceptible to injury than slowly growing ones. It has been suggested that only the time of action of an agent determines the kind and degree of maldevelopment, regardless of the nature of the agent. If this were true, all malformations should fall into one spectrum of forms determined by time and intensity of the damage. We know that this is not the case and that many agents produce characteristic abnormalities within a considerable range of time, whereas patterns of growth and sensitivity may change rapidly during periods of organogenesis. The observation of external features of human embryos during the first two months of development will convince anyone that growth patterns must change from day to day. On the other hand, such infectious agents as rubella produce a narrow range of malformations over a period of several weeks or months. It must be concluded that both the time of action and the nature of the agent determine the characteristics of abnormal development. In some instances the one aspect is more obvious, and in others the other, but both affect the outcome in every case.

Just as it is fallacious to consider the timing of an insult as the sole determinant of the type of malformation produced, it is also inconsistent with experience to postulate that adverse conditions affect the embryo by means of a single mechanism, i.e., "vascular disorders induced by hypoxia."[86] Here again, the variety of pathogenic mechanisms shows that uniform effects cannot be reasonably anticipated.

The assumption that one may, on the basis of descriptive human embryology, determine the latest stage at which a given defect must have been determined (*teratogenetischer Terminationspunkt*)[87] is justifiable in only a limited number of instances. It may be applied with reason in such cases as transposition of the great vessels where the developmental plan must have been faulty when the aorta and pulmonary artery first formed as separate vessels. In other instances, however, reasoning of this kind is not permissible since it would not take

into account the possibility that regressive changes may have played a part. Another source of error is the fact that organogenesis occurs during a limited period of development. The component parts of many kinds of multiple malformations may be traced speculatively to that period, and then show amazingly good conformity of their hypothetical timing. This has recently been attempted in speculations about mongolism[38] when abnormalities which were thought (not observed) to arise "about the forty-second day," "about the fifty-seventh day," "late in the second or early in the third month" and "as early as the third and fourth fetal months" were combined to show the predominant significance of timing of an insult, as if the pattern of sensitivity of the embryo were the same at all these times. The conclusion was reached that these embryos "weathered a period of critical, almost lethal stress" and that mongolism is probably "a stage specific defect of about the eighth week of fetal life." The number of doubtful and unjustified steps in this reasoning is so great that the conclusions reached have no value even as a working hypothesis.

Another concept which has been promoted in recent years is that of the status Bonnevie-Ullrich. It is based on a study of defects of the eyes and extremities faintly similar to those described here, occurring among other malformations in a mutant strain of mice. Bonnevie[39] claimed that cerebrospinal fluid escaping in increased amounts from the brain, travels in the form of blebs under the epidermis and causes abnormalities where the blebs become stationary. Ullrich[40,41] explained numerous congenital defects by the same mechanism without the slightest evidence for his contention. Actually, it is almost certain that Bonnevie's account of the traveling blebs is erroneous.[23] Furthermore, numerous investigations, some of which are quoted above, indicate in well documented form the existence of other mechanisms such as necrosis of previously well formed parts. The status Bonnevie-Ullrich has no basis either in fact or in deduction. Some of the congenital defects classified under that heading are due to regressive changes as described here or to sequelae of vascular changes resembling those produced by pressor substances (*acroblapsie*[23]).

A third concept which has appeared in the literature of teratology, the classification of malformations as typical or atypical, should be examined critically in the light of the knowledge of regressive changes in the embryo. Szily[42] characterized "typical" malformations as those which follow a characteristic developmental process and regularly lead to the same end result. He stated that in these instances it should be possible to recognize a series of developmental stages, and that in early

stages the appearance of the affected primordia should not be that of diseased tissues. Furthermore, "typical" malformations should be inherited according to mendelian laws. Subsequently, Politzer and Sternberg[48] classified as "typical" those malformations in which only one organ or region is affected; each of the cases exhibiting a "typical" malformation should resemble the others. These authors believed that the malformations are frequently hereditary. According to both classifications, "atypical" malformations are those which fail to have such characteristics. Since the appearance of these papers, it has been learned that malformations caused by environmental factors may appear in "typical" forms. The example of selenium-induced abnormalities, at least in their early stages, is in point. Here, as well as in many hereditary malformations, there is evidence indicating the existence of abnormal tissue during early morphogenesis of the anomaly. This is in contradiction to one of Szily's postulates. There are obviously "typical" and "atypical" malformations as judged by the presence or absence of uniform features in the early or final stages, but neither category bears a distinct relationship to the genetic or environmental cause, or to one or another form of developmental mechanism.

Summary

Selenium compounds fed to hens and transmitted to eggs before laying, produced abnormalities in chick embryos which in their early stages showed a remarkably constant pattern of tissue necrosis. Recently published work contains many other examples of similar regressive lesions leading to malformations of the embryo. This feature is emerging as one of the important mechanisms in the pathogenesis of malformation.

The changes in the embryos of selenium-fed hens have been described in detail for the first 5 days of incubation. Necrosis appeared after 2½ to 3 days of incubation in certain areas of the brain, spinal cord, eyes and limb buds. As a result, there were not only defects in these organs, but structural alterations in nearby areas. The latter included the face, the nasal pits, the upper beak, and the caudal portions of the embryo developing from the trunk-tail-node. There was marked retardation in growth and differentiation of directly or indirectly affected primordia. In addition, an overall retardation of growth was evident by the fifth day. No embryos older than 5 days were studied. The manner in which processes of healing and partial restitution might contribute to a variable final outcome, therefore, could not be investigated.

The significance of regressive changes similar to those described

here, has been discussed. The observations indicate that it is necessary to re-evaluate a number of concepts purporting to explain the mechanism of malformation. Among these are the *teratologische Terminationspunkt* of Schwalbe, the classification of malformations as typical or atypical, and the status Bonnevie-Ullrich, which appear to be unacceptable in the light of the studies reported.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

Sections of a 3-day embryo (# 5). This is the earliest stage at which characteristic abnormalities were consistently found.

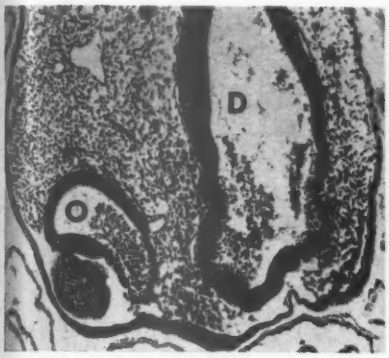
FIG. 1. Head, showing diencephalon (D), optic cup (O), and lens (L). Necrosis is evident in parts of the diencephalon and optic cup. The optic ventricle is much larger than normal (compare with Fig. 8). Mallory's trichrome stain. × 84.

FIG. 2. Higher magnification of part of Fig. 1. Mallory's trichrome stain. × 253.

FIG. 3. Wall of rhombencephalon with pyknotic nuclei. Mallory's trichrome stain. × 1,185.

FIG. 4. Spinal cord with pyknotic nuclei. Mallory's trichrome stain. × 1,185.

FIGS. 5 and 6. Caudal portions of spinal cord (C) and somites (S), showing areas of necrosis. Mallory's trichrome stain. × 348.



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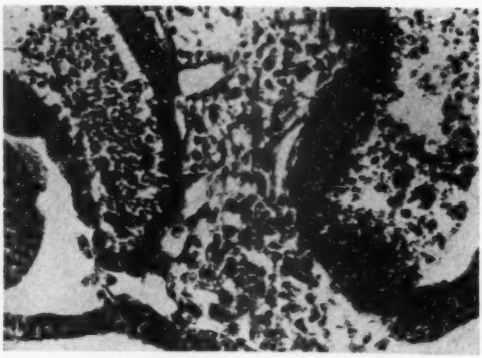

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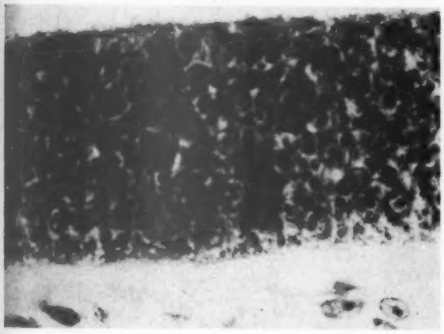

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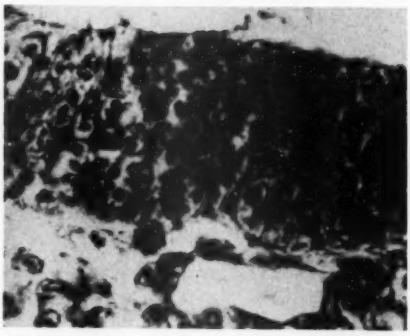

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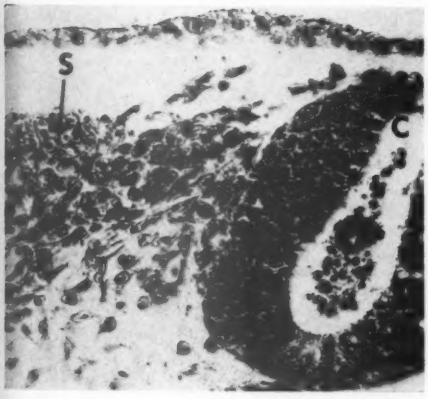

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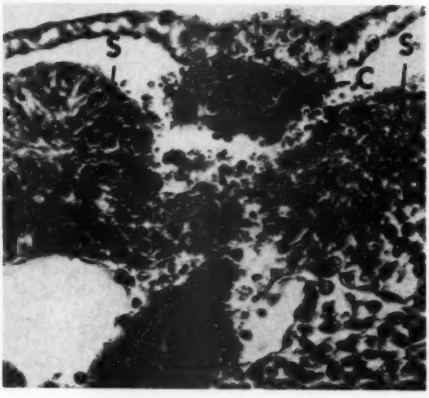

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Sections of a normal 2½-day embryo, for comparison with other figures.

FIG. 7. Section of head with telencephalon and nasal pits (N). Hematoxylin and eosin stain. × 101.

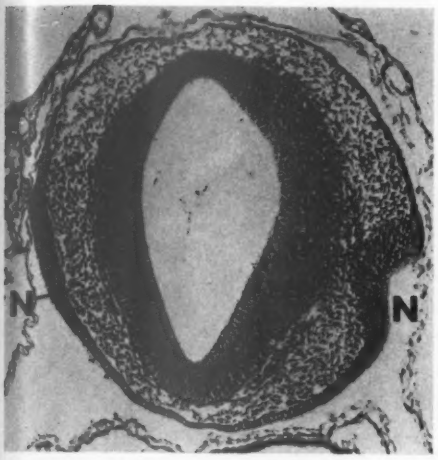
FIG. 8. Section of head with diencephalon (D), optic cup (O), and lens (L). Hematoxylin and eosin stain. × 101.

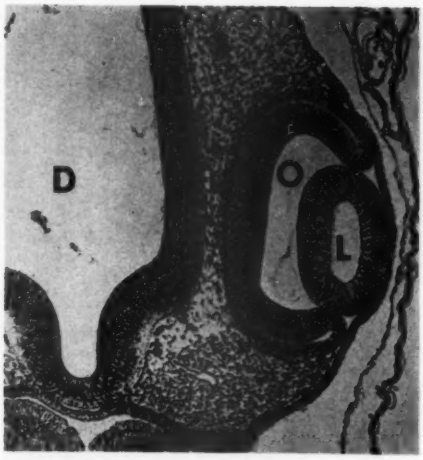
FIG. 9. Section of tail with spinal cord (C) and mesenchyme, showing beginning differentiation of notochord (A). Somites have not yet differentiated at this level. Hematoxylin and eosin stain. × 101.

FIG. 10. Rhombencephalon (R) of abnormal 5-day embryo (# 3), with hemorrhage near point of emergence of cranial nerve. Hematoxylin and eosin stain. × 138.

FIG. 11. Section of the same embryo at the level of the leg buds (B). The spinal cord is absent and the leg buds are nearer the midline than normal. Mallory's trichrome stain. × 78.

FIG. 12. Part of section of tail of 4-day embryo (# 5) with normal notochord (A) and somites (S). The primordium of the spinal cord (C) is reduced to a small, solid cell group. Mallory's trichrome stain. × 184.





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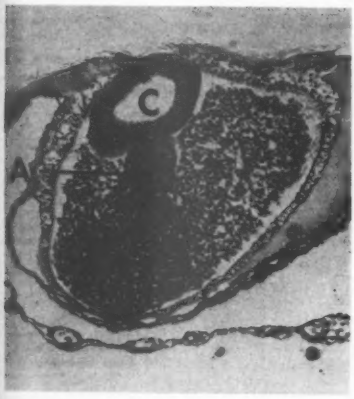

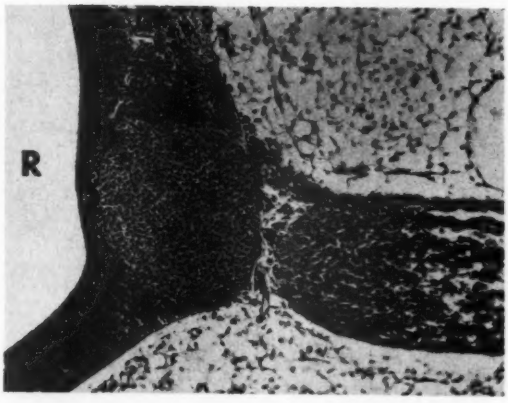

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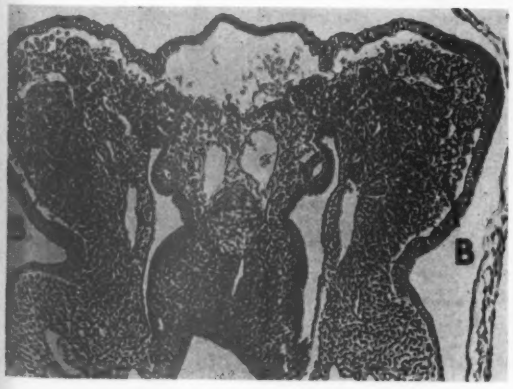

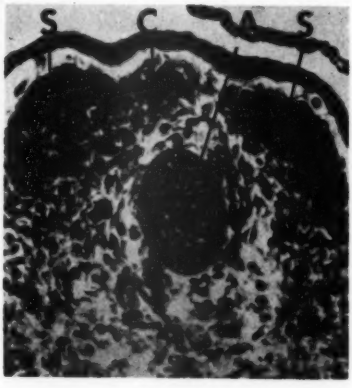

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Abnormalities of nervous system, eyes and tail.

FIGS. 13 to 16. Sections of a 4-day embryo (# 2). This embryo was reconstructed (Text-fig. 2). Hematoxylin and eosin stain.

FIG. 13. Head with telencephalon. In the areas where the hemispheres should develop, the wall is abnormally thin and remnants of necrotic tissue are attached to it (arrows). A tangential section of the optic cup is seen on one side. The nasal pits (N) are small, and are located more ventrally and medially than usual. × 73.

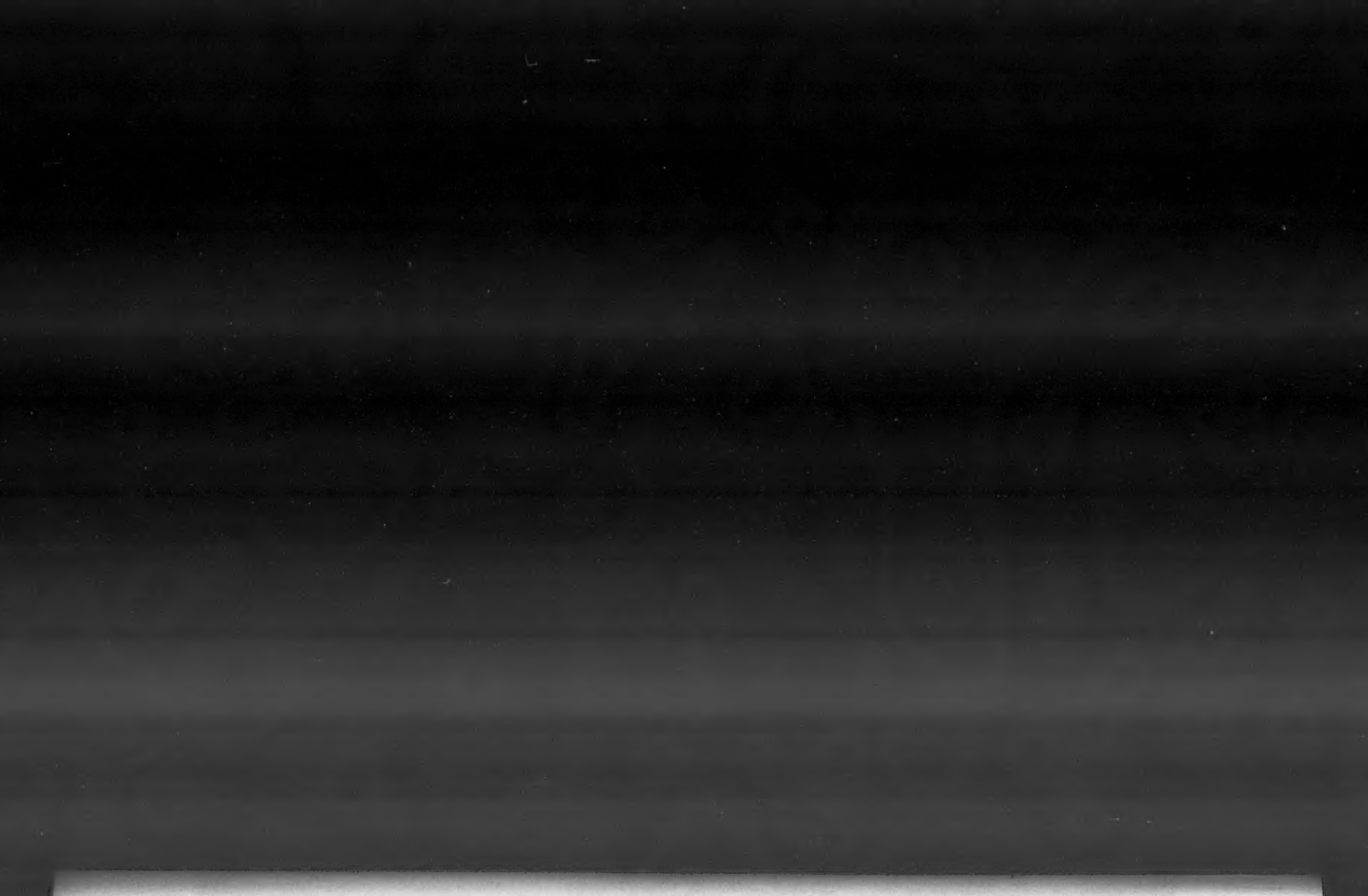
FIG. 14. Diencephalon, showing extensive necrosis. × 151.

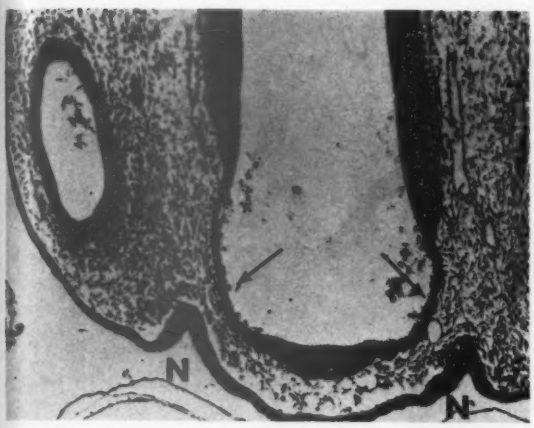
FIG. 15. Spinal cord. The lateral portions are completely destroyed. × 86.

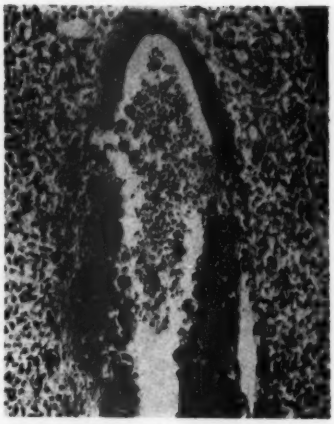
FIG. 16. Tail with well developed somites (S). A remnant of spinal cord cannot be definitely identified between the somites. × 430.

FIG. 17. Part of a section of a 5-day embryo (# 4). The spinal cord (C) is greatly reduced in size, but the spinal ganglia (G) are well formed. (A) is notochord. Mallory's trichrome stain. × 430.

FIG. 18. Part of diencephalon (D), optic cup (O), and lens (L), of a 5-day embryo (# 2). All show necrosis. The tissues of the diencephalon and optic cup are greatly reduced in thickness. Optic cup and lens have abnormal cavities. Hematoxylin and eosin stain. × 151.







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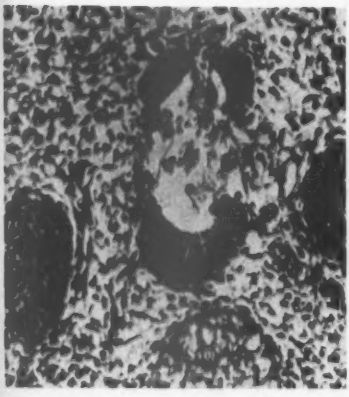

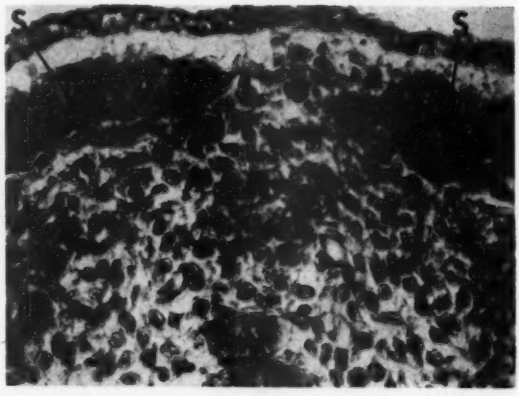

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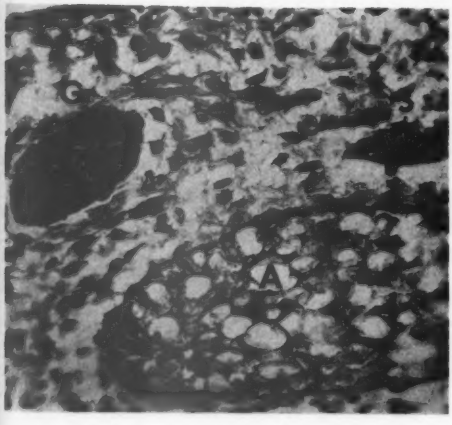

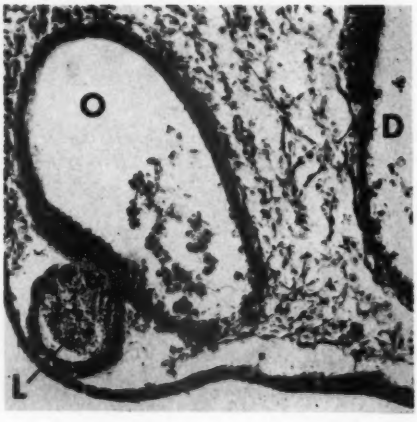

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Lesions in the limb buds.

FIG. 19. Leg bud of 5-day embryo (# 3), showing pyknosis and phagocytosis of nuclei and a clear space under the epidermis as evidence of necrosis. Hematoxylin and eosin stain. × 122.

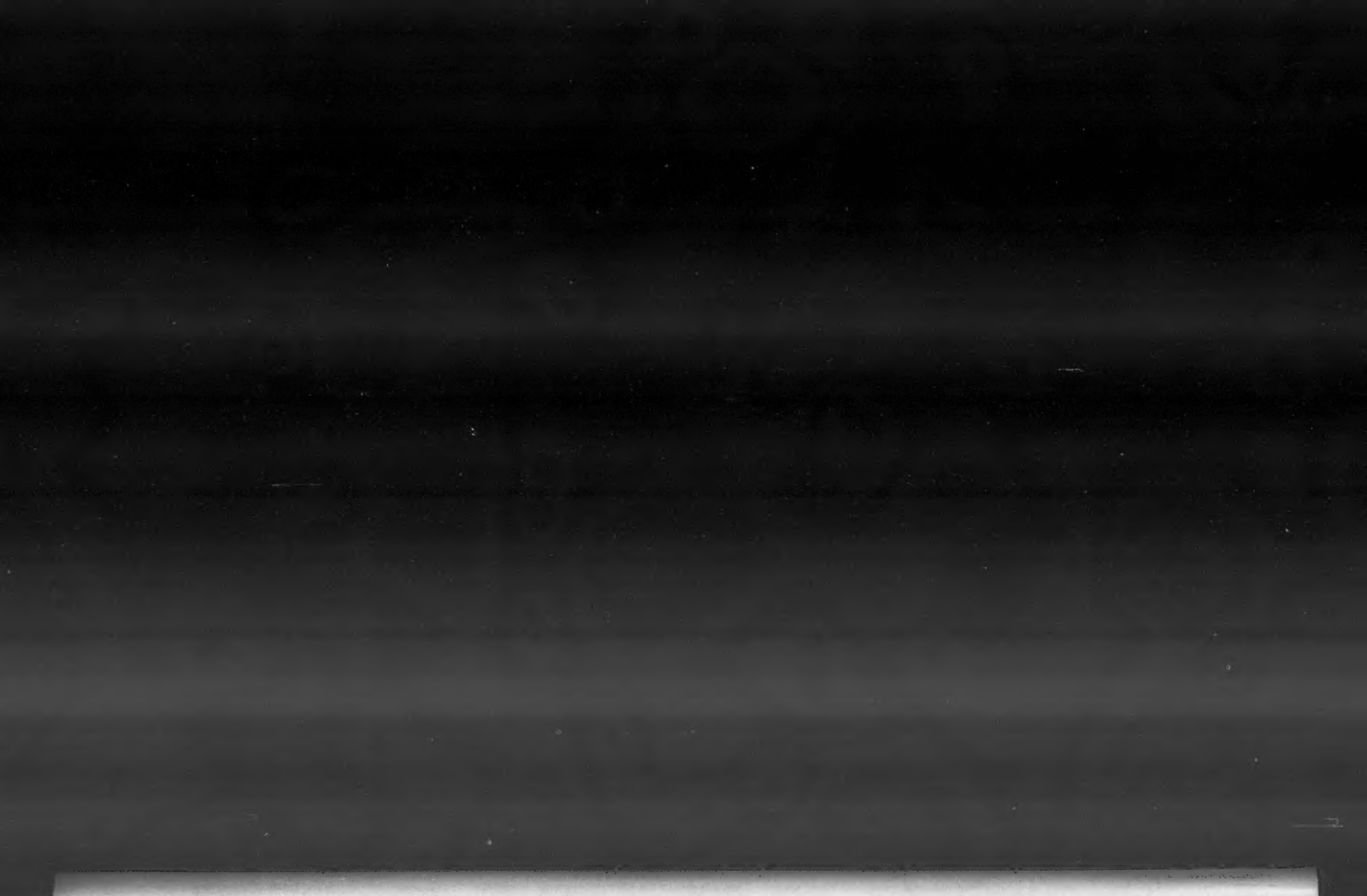
FIG. 20. Higher magnification of part of Fig. 19. Hematoxylin and eosin stain. × 326.

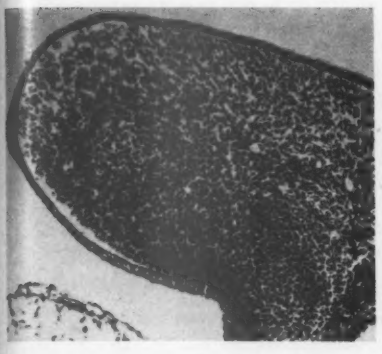
FIG. 21. Wing bud of a 4-day embryo (# 3) with extensive necrosis in the mesenchyme. Mallory's trichrome stain. × 143.

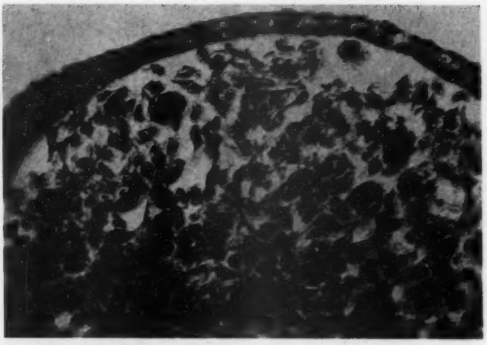
FIG. 22. Higher magnification of part of Fig. 21. Mallory's trichrome stain. × 326.

Late stages of lesions in embryos from eggs injected with selenium compounds.

FIGS. 23 and 24. Sections of an embryo of the second week, showing deformed cerebral ventricles (V), reduced hemispheres (H), and microphthalmia (O). Mallory's trichrome stain. × 20.







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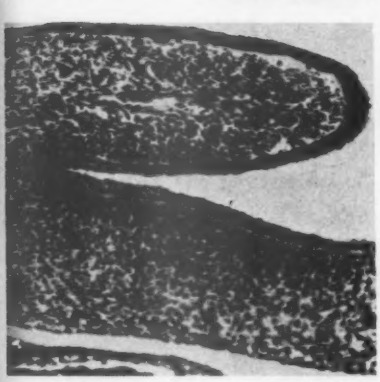

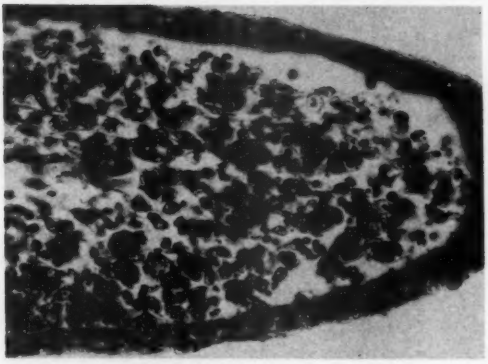

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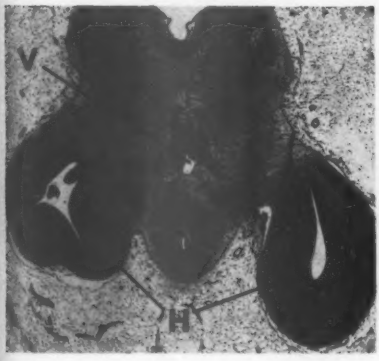

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GASTRIC LESIONS IN EXPERIMENTAL ANIMALS FOLLOWING SINGLE EXPOSURES TO IONIZING RADIATIONS *

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Past studies indicate that the stomach is less sensitive to ionizing radiations than the small intestine. In the intestine, functional changes have been noted within minutes,[1] and inhibition of mitosis is evident within hours of exposure to low dosages of whole body irradiation. Lethal† dosages produce partial denudation of the intestinal mucosa, but regeneration is usually complete within a week. At supralethal doses, complete loss of epithelium from the small intestine leads to extensive fluid loss and, in most species, to death in 3 to 4 days.[2,3] Guinea pigs and hamsters differ according to the propensity of the remaining epithelial cells to stretch out and cover the flattened villi.[4] The resulting reduction in loss of fluid lengthens survival to 5 to 6 days. However, the basic process of extensive destruction of the epithelium of the small intestine is similar in all species studied. The lesions represent the direct effects of radiation, since they can be reproduced by single local exposures of the abdomen or the intestine itself.[5]

No similar early lesions have been observed in the stomach following single exposure of the whole body to radiation. Changes in gastric motility have been noted.[6] Anatomic lesions and alteration in gastric secretion have not been described except after local and usually multiple exposures with cumulative doses greatly in excess of those that will produce severe lesions of the small intestine.[7-11]

Recently, plasma transfusion, marrow transplants and the administration of antibiotic agents have prolonged the survival of dogs,[2] rats[12] and hamsters,[4] following exposures that killed unprotected animals in 3 to 6 days. In these animals gastric lesions developed 2 to 3 weeks after irradiation. Following the single exposures, the time sequence of the development and healing of gastric lesions was studied and compared with the lesions of the small intestine in the same animals. The complex conditions of multiple dosages, with different rates of partial recovery of various tissues between exposures were avoided.

* Received for publication, May 28, 1957.

† The term "lethal" is used for doses that kill 1-99 per cent of animals, "supralethal" for uniformly killing doses.

Material and Methods

Dogs were given whole body irradiation by a Co^{60} source with 1,200 to 1,700 r. Of 11 dogs given electrolytes, plasma, and protein hydrolysates by infusion, 4 survived 6 to 12 days.[2] Twenty dogs without such infusion died 3 to 4 days after exposure from acute dehydration and vascular collapse. In another experiment, 6 dogs were given local irradiation to the stomach, the source being a 200 KVP Picker therapy roentgen machine (25 ma, 1 mm. Al, 0.15 mm. Cu, TSD 50 cm., HVL 0.8 mm. Cu, dose rate in air 44 r/min). Radiation was applied with or without eventration of the stomach through a midline incision, followed by replacement of the stomach and surgical closure. In 2 of the dogs biopsy specimens of the gastric mucosa were taken 3 weeks later, and 4 dogs were sacrificed between 2 and 4 weeks after exposure.

Rats, too, were given roentgen irradiation; the machine qualities are described above. Within an hour of exposure to 800 or 900 r., the rats were joined in parabiosis with nonirradiated litter mates.[12] Single irradiated animals died in 3 to 5 days. Parabiosis prolonged survival of 50 per cent of the animals to 30 days or longer.

Hamsters were exposed to 1,160 r. with a clinical roentgen machine operating at 200 KVP (20 ma, HVL 0.76 cm. Cu.). Some of these animals had one leg shielded during irradiation. Others received an intracardiac injection of one homogenized spleen and the marrow from 4 long bones of a 2-week-old normal hamster within a few hours of irradiation.[4] Approximately half of the animals so treated survived longer than 30 days while all animals not receiving the spleen and marrow homogenate died in 4 to 8 days.

Histologic studies were made on animals that died spontaneously or were sacrificed. Both those receiving protective substances and those not so treated were included. Tissues were fixed in buffered formalin sublimate solution[18] and paraffin sections were stained with hematoxylin and eosin.

Results

Dogs

Gastric and intestinal lesions were compared 3, 6 and 10 to 12 days after whole body exposure to 1,200 r. At 3 days, the architecture and appearance of individual cells of the fundic and pyloric mucosa of dogs were normal (Figs. 1 and 2). In contrast, the mucosa of the small intestine was extensively denuded. The remaining lining cells were stretched out and flattened in an apparent attempt to maintain continuity of the epithelium (Fig. 3). Cells with enlarged vesicular nuclei

and prominent central nucleoli were conspicuous. At 6 days only one dog was available for study. Its fundic mucosa was normal. In the pyloric mucosa the base and pits of the glands were unchanged but the mid thirds were lined by hyperchromatic cells with centrally located, occasionally swollen nuclei and prominent nucleoli reminiscent of early radiation changes in the small intestine. These altered cells were closely packed, with no significant diminution in number (Fig. 4). At that time the mucosa of the small intestine had been reconstituted. In most areas, a moderate hyperplasia of the lining epithelium was the only remaining abnormality (Fig. 5). In a few areas, hyperplastic crypts and glands alternated with others lined by the flattened, stretched-out cells seen earlier (Fig. 6). Ten to 12 days after receiving 1,200 r., the fundic glands were partly denuded of epithelium and the remaining cells were hyperchromatic, with swollen nuclei and, often, distinct central nucleoli (Fig. 7). Some parietal cells could be identified by their eosinophilic staining quality. Occasional mitotic figures were present, some of them of abnormal nature. Similar changes were seen in the pyloric mucosa. At this time the mucosa of the small intestine was fully regenerated and indistinguishable from that of control dogs (Fig. 8). No lesions were found in the mucosa of the colon of the dogs in this series.

None of the dogs given 1,200 r. whole body irradiation were kept alive longer than 12 days. Consequently the later fate of the gastric lesion could not be followed in this group of animals. The gastric mucosa was therefore studied after local irradiation with 1,200 to 1,700 r. Dogs so irradiated generally continued in good health and did not suffer from the diarrhea attending denudation of the small intestine following whole body irradiation. Anorexia was noted in one of the dogs.

Three dogs sacrificed 2 weeks after the local exposure to 1,700 r. showed lesions of the fundic glands similar to those described following whole body irradiation with 1,200 r. The lumens of many of the glands were widened because of flattening of the lining cells. Many of these cells were eosinophilic. Polymorphonuclear leukocytes were present in some of the glands. Occasional foci of hyperchromatic cells with scattered mitotic figures were present at the level of the foveolas, apparently representing early attempts at regeneration. Regeneration was more prominent, however, in the pyloric region (Fig. 9), again originating in the pits of the glands. The cylindrical cells with basal nuclei characteristic of the pyloric glands were well preserved in some

glands, whereas in others transition to cuboidal cells with more centrally located nuclei was seen. In 3 of the 4 dogs on which biopsies were performed or which were sacrificed at 21 days, regeneration of the fundic and antral epithelium proceeded from the neck of the glands where the rare mitotic figures in normal mucosa are located. However, no regeneration was evident in one dog in a biopsy specimen procured at 21 days. Extensive local ulceration had developed (Fig. 10). The few remaining groups of epithelial cells were arranged in an acinar pattern. The individual cells were large, with "owl eye" nuclei. When this dog was sacrificed 32 days after irradiation, a wide area at the margin of the ulcer was covered with a single layer of epithelium, except for a few newly formed hyperplastic glands. The very abnormal cells seen in the 21-day biopsy specimen were no longer present.

Rats

Sections were made from the stomachs of 3 rats sacrificed or found dead 3 weeks following exposure to 800 or 900 r. and subsequent experimental parabiosis. There was spotty loss of cells with flattening of the remaining epithelium in many of the gastric glands. A prominent feature was the presence of hyperplastic epithelium with frequent mitotic figures in the neck of some of the glands (Fig. 11). Complete repair was prompt. No gastric lesions were seen in animals surviving 40 days or longer. Sections were also made from the tissues of numerous animals which died during the first 2 weeks after exposure to 800 and 900 r. No convincing evidence of damage to the gastric epithelium was revealed. There were focal dilatation of glandular lumens and apparent loss of the epithelium, but these conditions were occasionally found in controls and were often indistinguishable from artifacts. The small intestine, as in dogs, underwent rapid denudation and re-epithelization with complete restitution by the end of 7 to 10 days.

Hamsters

The time sequence of injury to the small intestine and recovery was similar to that in rats and dogs. Gastric lesions were not seen during the first week following exposure to 1,160 r. At 3 to 4 weeks, however, lesions were found in the glandular stomach of each of 8 animals examined. Hyperplastic epithelium with frequent mitotic figures was always present in at least some of the gland pits. Occasionally, large areas exhibited this clear-cut evidence of regeneration. There were ulcerations in some stomachs, suggesting failure of regeneration (Fig. 12). In one experiment, adequately preserved tissues were secured from 10

animals dying between the 28th and 56th days. In 7, gastric ulcers were present and were probably the cause of death. In another series, very few deaths occurred during the 2nd and 3rd month after similar exposure and treatment, indicating that lethal progression is not a necessary sequela of the gastric ulceration.

Discussion

Inhibition of mitotic activity and death of cells as they enter the next mitosis are presumably the common features of radiation damage in both the stomach and small intestine. A detailed review of the pathogenesis of the intestinal lesions has recently been published by Quastler.[14]

In the small intestine the crypt cells provide the reservoir of regenerating epithelium. The few Paneth cells at the very bottom of the crypts and the goblet and chief cells covering the villi represent the progeny of these formative elements. The life span of the mature cells is estimated at 36 hours, the oldest cells being shed into the lumen from the tip of the villus, while new cells gradually migrate up its sides. Denudation of the small intestine would be expected within 1½ days of cessation of mitotic activity. This is postponed, however, by failure of the usual migration upward.[15] Old cells cling to the shortened villi upon which they stretch out, thus providing a continuous epithelial lining.* Differences in the efficacy of this clinging property may account for differences among species. In rats denudation is complete 3 days following exposure to 1,000 r., but in golden hamsters complete denudation is absent as late as 6 days even after the application of 4,000 r.

Quastler's thesis[14] is presumably applicable to the gastric mucosa. In the fundus, 4 types of cells can be distinguished, the mucus-secreting surface epithelial cells, the neck cells, the parietal cells and the chief or zymogenic cells of the tubular glands. The surface epithelium is replaced by dividing cells in the pits of the glands. The neck cells, contiguous with but differing from the first type of mucus-secreting cells, also divide, but the parietal and chief cells probably do not.[16]

The arrest of mitosis by colchicine has been used to determine the number of cells entering division in a given time. In the rat stomach, Stevens and Leblond[17] found 2.6 per cent of mucous neck cells and 5.9

* Recently, H. Quastler and F. G. Sherman studied radioautographs of mouse small intestine after labeling of deoxyribose nucleic acid by incorporating tritium into thymidine. In these as yet unpublished experiments the migration out of the generative zone was not appreciably modified by irradiation, and the "clinging ability" of the epithelium appeared to be the major feature which increased survival of the cells and delayed denudation and death.

per cent of surface epithelial cells in mitosis 4 hours after colchicine administration. From these data they computed the renewal times of surface epithelial cells as 3 days and of mucous neck cells as 6½ days. The computations presuppose that the cells replace only themselves.

There is considerable support, however, for the thesis of Bizzozero[19] that only the cells in the pit and neck of the glands divide, and that both the surface epithelium and the parietal and chief cells regenerate from these formative cells. The present observations tended to confirm this theory. Areas of regenerative hyperplasia were prominent in the neck of the glands. It is not known, however, what the normal life span of parietal and chief cells is, or whether they are replaced only in response to injury. If the neck cells, which are greatly outnumbered by the chief and parietal cells, replace them continuously, then the life span of chief and parietal cells must be much longer than the 6-day renewal time computed for the neck cells themselves. Since the survival of surface epithelium following irradiation considerably exceeded the 3-day life span suggested by Stevens and Leblond, there must be retention of cells that would normally be shed and replaced. This would also apply to the pyloric mucosa for which Leblond and Walker[16] suggested renewal times of just under 2 days. The apparent prolongation of life span would be comparable to the greater average age of automobiles in wartime due to nonavailability of new ones. Alternatively, the renewal time as derived from observation of mitotic activity may underestimate average life span. The mitotic index as a measure of cell renewal may be likened to the reticulocyte count as an indicator of production of red cells. Estimates of red cell life span from reticulocyte data are notoriously unreliable.

The development of gastric lesions in experimental animals was comparable to the lesions observed in serial gastric biopsy specimens from 3 patients with duodenal ulcers who received a fractionated total exposure to 1,600 r. to the fundus of the stomach.[18] The delay in actual loss of damaged cells, the appearance of swollen cells with abnormal nuclei and nucleoli, and the hyperplastic regeneration proceeding from the pits of the glands were common to both the human and experimental lesions. In contrast to the present experiments, the delay in the onset of the lesions of human subjects was less significant since the irradiation extended over a 10-day period. Of greater interest was the observation of coagulation necrosis in the depth of the tubular glands of the human fundus and the very slow repair. Coagulation necrosis was noted early but persisted nearly 5 weeks in 2 of 3 patients. Differences between species, variation in depth dose, and the utilization

of single or fractionated exposures to irradiation could play roles in these varied responses.

The appearance of more atypical cells 2 to 3 weeks following irradiation may have represented the late development of degenerative changes, or these cells may have undergone one division or more and then assumed bizarre shapes during unsuccessful attempts at further division. The second possibility is suggested by the observation of undifferentiated cells with vesicular nuclei and occasional abnormal mitotic figures lining the entire length of tubular glands (Fig. 6). Degeneration *in situ* incident to entering the next mitosis appears unlikely, since fully differentiated parietal and chief cells do not normally divide. It appears more likely that these cells represented recent progeny of damaged neck cells incapable of continued normal development.

Progression of the gastric lesion to frank ulceration was observed occasionally in hamsters after full recovery of bone marrow function. It occurred after local irradiation of one dog with normally functioning bone marrow. We have pointed out previously that regeneration of small intestine epithelium following whole body irradiation is usually complete by the time dogs develop pancytopenia and die from infection and hemorrhage.[3] Ulceration of the gastrointestinal tract secondary to hemorrhage and agranulocytosis may occur at that time. In dogs, the tonsils and anal mucosa are usually diseased. In guinea pigs the stomach is frequently the site of hemorrhagic lesions which may ulcerate. "Agranulocytic ulcers" of the small intestine have been noted in this laboratory in mice, rats, and hamsters. These lesions were clearly distinct from those representing the delayed but direct radiation damage to the gastric mucosa here described.

Summary

Epithelial changes in the gastric mucosa developed about two weeks following single supralethal exposures to irradiation. These changes were quite similar to those which occur earlier in the small intestine. Regeneration proceeded from the neck of the glands and was comparable to the regeneration of small intestine mucosa from surviving crypt epithelium. Occasionally, the direct damage was severe enough to lead to ulceration, even after local irradiation and in the absence of agranulocytosis and hemorrhage. This was in contrast to the late ulcers appearing in the regenerated mucosa of the small intestine which were associated with hemorrhage or represented direct sequelae of the pancytopenia that follows lethal irradiation.

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[*Illustrations follow*]

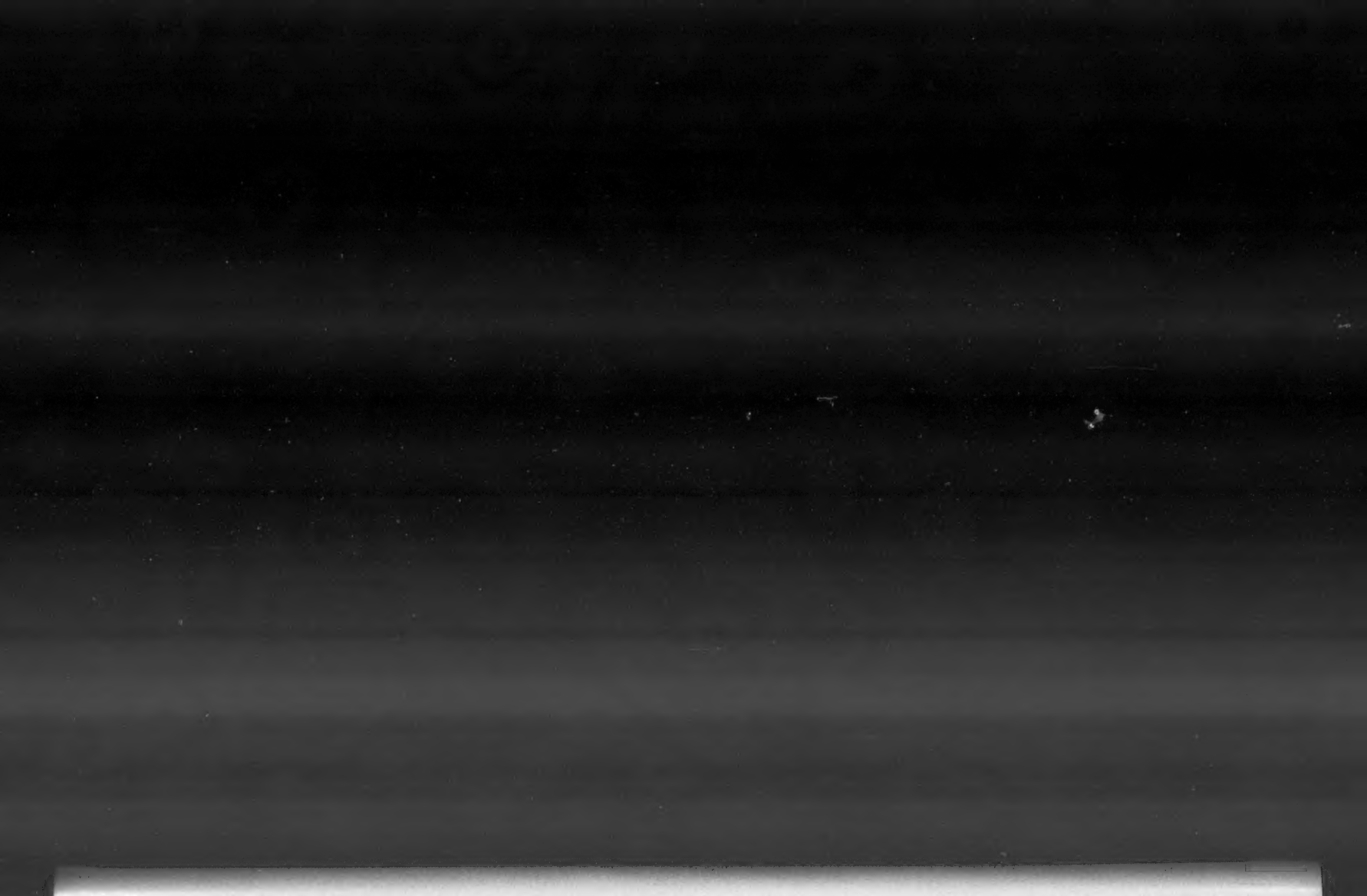
LEGENDS FOR FIGURES

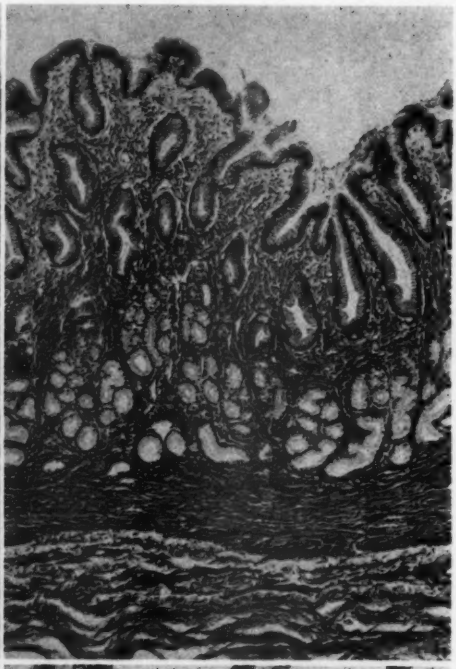
FIG. 1. Dog. Fundus of stomach. Normal appearance 3 days after receiving 1200 r. Hematoxylin and eosin stain. × 38.

FIG. 2. Dog. Pylorus. Normal appearance 3 days after receiving 1200 r. Hematoxylin and eosin stain. × 38.

FIG. 3. Dog. Small intestine, showing loss of epithelium with remaining cells flattened. Hematoxylin and eosin stain. × 38.

FIG. 4. Dog. Pylorus 6 days after exposure to 1200 r. Early lesion. Hematoxylin and eosin stain. × 50.





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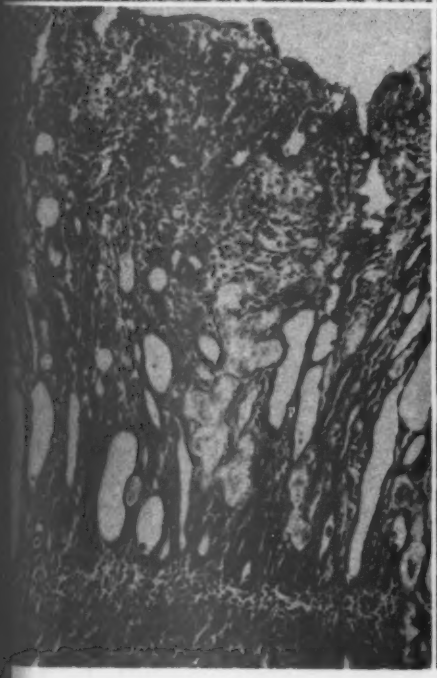

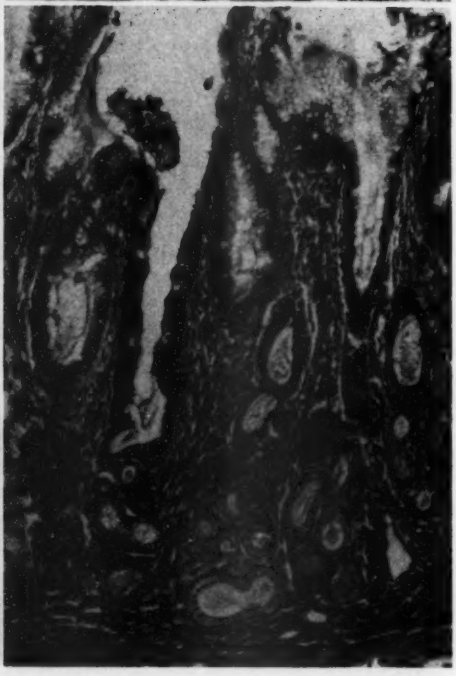

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FIG. 5. Dog. Small intestine. Slightly hyperplastic, regenerating epithelium 6 days after exposure to 1200 r. Hematoxylin and eosin stain. × 38.

FIG. 6. Dog. Small intestine, revealing delayed regeneration compared with area shown in Fig. 5. Hematoxylin and eosin stain. × 38.

FIG. 7. Dog. Partial destruction of fundic glands 11 days after irradiation with 1200 r. Hematoxylin and eosin stain. × 80.

FIG. 8. Dog. Fully regenerated small intestine. Hematoxylin and eosin stain. × 38.

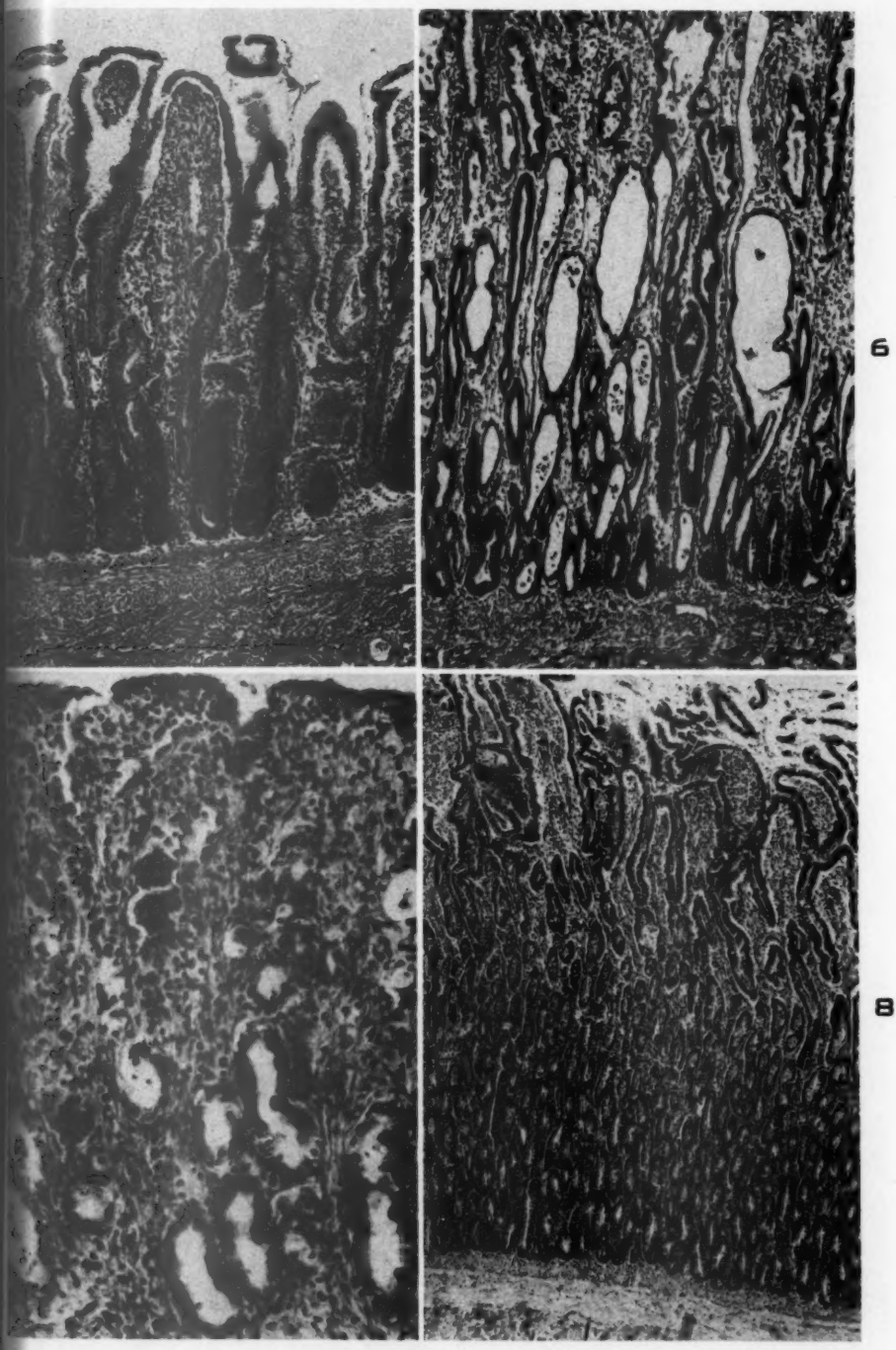

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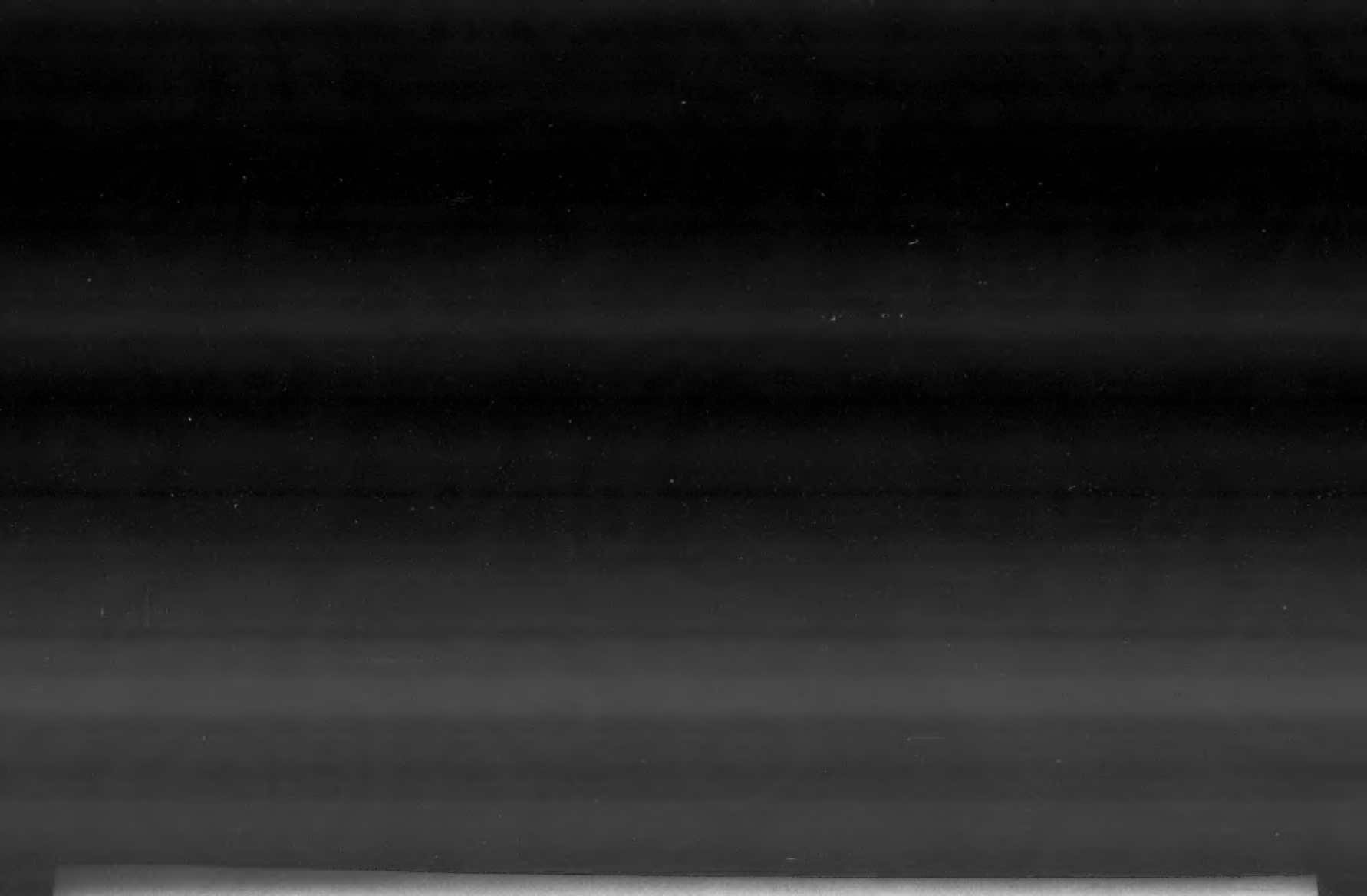
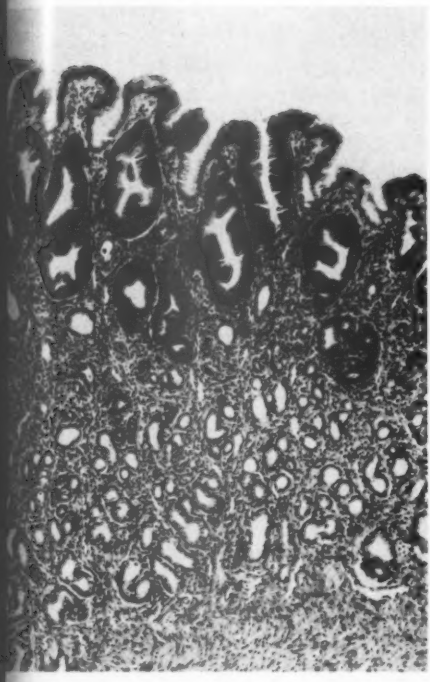
FIG. 9. Dog. Hyperplastic, regenerating foveolas of the pylorus, 14 days after local irradiation with 1700 r. Hematoxylin and eosin stain. × 38.

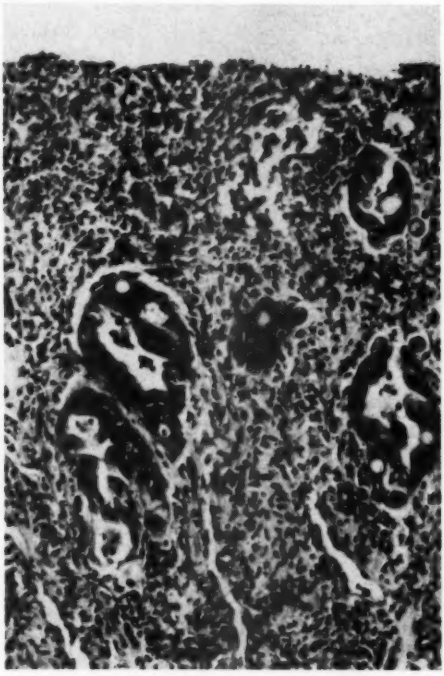
FIG. 10. Dog. Biopsy of stomach 21 days after local irradiation with 1700 r. Hematoxylin and eosin stain. × 80.

FIG. 11. Rat. Hyperplastic neck glands of stomach, 21 days after exposure to 900 r. Hematoxylin and eosin stain. × 50.

FIG. 12. Hamster. Ulceration and regeneration of gastric mucosa, 21 days after irradiation with 1150 r. Hematoxylin and eosin stain. × 38.







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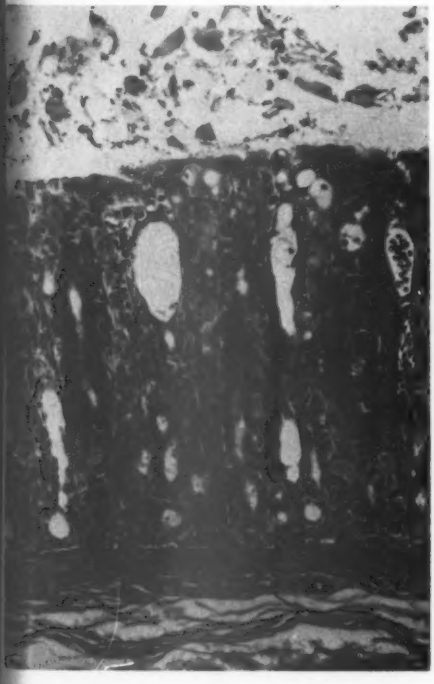

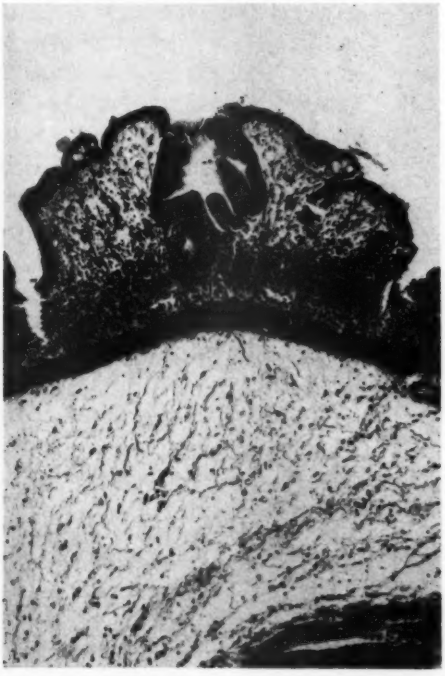

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THE EFFECT OF VARIOUS DIABETOGENIC HORMONES ON THE STRUCTURE OF THE RABBIT PANCREAS*

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It has been demonstrated previously that cortisone administered to rabbits causes diabetes mellitus of variable severity, accompanied by degranulation of B cells and glycogenization (vacuolization) of pancreatic ductular epithelium and B cells. Diffuse and focal proliferation of ductular epithelium was also observed.[1-3] Loss of B cell granulations and B cell vacuolization are well known concomitants of most forms of experimental diabetes mellitus.[4-6] The B cell vacuolization was thought to be an initial alteration proceeding to the destruction of these elements, to insulin deficiency and ultimately to permanent experimental diabetes. This pathogenetic mechanism was originally advanced to account for the permanent diabetes induced in dogs by partial pancreatectomy[7,8] and was invoked also to explain the metadiabetes produced in either normal or partially pancreatectomized dogs and cats by anterior pituitary extracts,[9-12] growth hormone,[13] adrenal steroids,[14] thyroid extract,[15] and glucose.[16,17]

Despite the fact that marked hydropic change was noted in the B cells of guinea pigs[18,19] and rabbits[1,2,20] after treatment by adrenal steroids, no evidence of B cell destruction or of metadiabetes has been reported in these species. It was, therefore, suggested that the duration of hydropic change had probably been insufficient for the development of B cell destruction in these species. This hypothesis is in accord with the fact that metadiabetes appears in the cat only after a considerably longer period of severe diabetes than in the dog.[11,12] Hydrocortisone has a greater effect on carbohydrate metabolism than cortisone.[21] It was, consequently, considered of value to determine whether this steroid would more consistently cause a greater severity and duration of hyperglycemia in the rabbit than cortisone and also a greater degree of hydropic change in its pancreas. Since previous workers suggested that purified growth hormone might act synergistically with hydrocortisone to increase the degree of diabetes in the rabbit,[22] the effect of simultaneous administration of these two hormones was studied. Moreover, it seemed desirable to determine whether endogenous steroid secretion stimulated by adrenocorticotropic hormone would also lead to diabetes and to the hydropic lesions as well.

* Received for publication, July 1, 1957.

Material and Methods

The study was carried out on 92 New Zealand white rabbits of both sexes weighing between 2,500 and 5,000 gm. Each animal was kept in a metabolic cage and fed Purina Rabbit Chow and water, *ad libitum*. The animals were weighed daily and blood was drawn from the marginal ear vein for evaluation of glucose. A modification of the Nelson-Somogyi micromethod for determination of glucose was utilized.[23] The rabbits were sacrificed by the intravenous administration of nembutal in lethal amounts at various time intervals up to 32 days. Originally, it had been intended to maintain the animals for longer periods of time. However, this was not feasible since many rabbits died during the course of the experiments. These are not included in this report. Necropsy was made promptly, and specimens of the tail of the pancreas were placed immediately in Zenker formol solution. Tissue was embedded in paraffin, and sections stained by a modification of the Masson trichrome stain,[24] the aldehyde fuchsin technique[25] with a trichrome counterstain, and the periodic acid-Schiff method for glycogen.[26] The latter was controlled by diastase digestion. Twelve rabbits served as controls; the others were divided into 3 groups.

Group A consisted of 38 rabbits, each of which was given 5 mg. per kg. of hydrocortisone acetate* intramuscularly daily up to the eighth day. After this time the dosage of hydrocortisone was increased to 10 mg. per kg. daily. Three animals of this group were sacrificed daily for the first 7 days; 2 animals were then sacrificed on alternate days up to the 21st day and one animal was sacrificed on the 24th, 26th and 31st days.

Group B consisted of 28 rabbits, of which 22 were given 5 mg. per kg. of hydrocortisone acetate intramuscularly and 1 mg. per kg. of growth hormone subcutaneously† daily. On each of the first 4 days and thereafter on alternate days up to the 18th day 2 rabbits were sacrificed. The other 6 rabbits of this group were given 5 mg. per kg. of hydrocortisone and 1 mg. per kg. of growth hormone for 5 days, after which the dose of growth hormone was increased to 2 mg. per kg. daily in an attempt to preserve the animals in a diabetic state for longer periods of time. Two animals were sacrificed on the 10th, 14th and 21st days.

Group C consisted of 14 rabbits. These were given corticotropin

* Hydrocortisone acetate was supplied through the courtesy of Dr. C. J. O'Donovan of the Upjohn Company, Kalamazoo, Michigan.

† Growth hormone was a gift from Endocrine Study Section, National Institutes of Health.

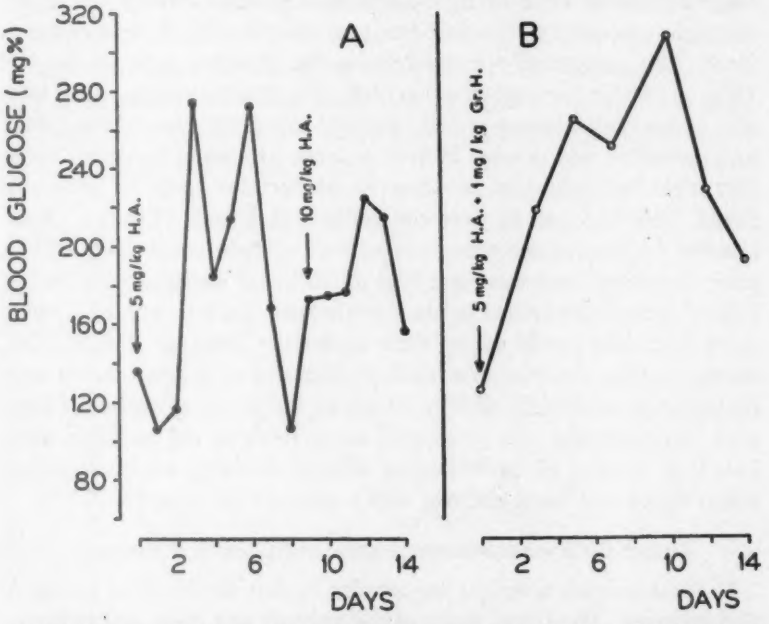
zinc* subcutaneously in daily doses which were gradually increased from 10 to 80 units over a period of 32 days. Two animals were sacrificed at 4-day intervals from the 8th to the 32nd days.

Results

Group A (Hydrocortisone Acetate)

The animals in this group usually developed marked weight loss within 10 days to 2 weeks after starting the administration of hydrocortisone. The weight loss was progressive and by the end of the experimental period the animals had frequently lost as much as 1,000 gm. In many instances animals died suddenly; usually the first deaths occurred at about 10 days after the initiation of treatment. At necropsy many rabbits had pneumonia or peritonitis.

Variable degrees of hyperglycemia were produced. Often, 24 hours after the first dose of hydrocortisone, there was a moderate elevation of the blood sugar to 175 to 200 mg. per hundred cc. and at 48 hours



Text-figure 1. Representative blood sugar curves of two rabbits. Rabbit A was treated with hydrocortisone acetate (H.A.). Rabbit B received H.A. and Growth Hormone (Gr. H.).

* Corticotropin zinc was supplied through the courtesy of Organon, Inc., Orange, New Jersey.

the blood sugar value frequently exceeded 200 mg. The maximum extent of diabetes was usually achieved by the 4th to the 6th day after which the blood sugar tended to decline. However, those rabbits receiving the increased amount of hydrocortisone exhibited another elevation of blood sugar and a secondary decline during the next week (Text-fig. 1A).

The pancreas contained the lesions which have been described following treatment with cortisone acetate. Evidence of B cell degranulation was first noted after 2 days. This became more marked as the severity of hyperglycemia increased, and frequently complete degranulation was observed after the 3rd or 4th day. No mitotic figures were observed in the B cells during the course of these studies. Glycogen appeared in duct cells on the 5th day, and in several instances it became marked by the 6th day. After that, glycogen was frequently seen in duct cells in large amounts while none was present in B cells (Fig. 1). In a few instances no duct cell glycogen was observed even after prolonged administration of hydrocortisone. A small amount of B cell glycogen was observed in an occasional animal after 8 days of treatment. This was usually located along the capillary pole of the cell (Fig. 2). Extensive vacuolization of B cells did not develop. Proliferation of duct epithelium was first observed in a rabbit treated for 5 days and thereafter was present in most animals. In many instances irregular islets and intra-islet proliferation of ductular epithelial cells was found. This appeared in close contiguity with B cells (Fig. 3). Occasionally inspissated secretion was seen to occlude small ductules. In other instances there was intra-islet dilatation of ductules with formation of spaces surrounded by duct epithelium and a corona of B cells. Some intra-islet cystic spaces were apparently lined by B cells. This seemed to have occurred as a result of dilatation of the duct lumen with compression and finally disappearance of the lining epithelium (Figs. 4-6). As treatment was prolonged, many lobes of the pancreas were found to consist of proliferating dilated ductules, dedifferentiated acinar tissue and comparatively intact islets (Figs. 7 and 8).

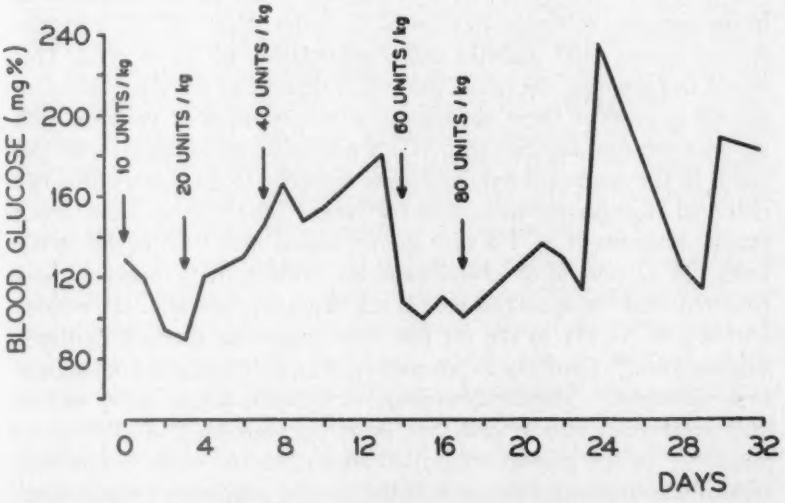
Group B (Hydrocortisone Acetate and Growth Hormone)

In these animals a weight loss similar to that described in Group A also occurred. Here, too, death of the animals was often due to infection. In general, the administration of the two hormones caused a greater severity of diabetes than hydrocortisone acetate alone. Hyperglycemia was observed more frequently after 24 hours and became quite marked by the 3rd or 4th day. The hyperglycemia was often

maintained until the 10th to 12th day after which there was a tendency for the blood sugar to return to normal levels (Text-fig. 1B). Concomitant with the increase in severity of diabetes, a more marked degranulation of B cells was observed. This also made its initial appearance after 48 hours. Duct cell glycogen deposits first appeared in animals sacrificed at 4 days and were usually marked by the 6th to 8th day. B cell glycogen was found after 8 days of treatment. No instance of very severe B cell hydropic change was produced and no evidence of destruction of these cells was observed. Ductular epithelial proliferation similar to that observed in Group A was also noted. This seemed to progress as the treatment was continued and did not appear to be related to the severity or duration of diabetes.

Group C (Corticotropin Zinc)

These rabbits only occasionally developed a mild form of hyperglycemia which declined rapidly and was only re-established with the administration of increased amounts of ACTH (Text-fig. 2). The pancreatic islets showed very moderate degrees of B cell degranulation. No glycogen was found in either ducts or B cells. Occasional mitotic figures were observed in the B cells of one animal treated for 8 days. In many instances, as in the other 2 groups, marked proliferation of duct epithelium was found.



Text-figure 2. Representative blood sugar curve of a rabbit treated with increasing dosages of corticotropin zinc.

Discussion

The degree of hyperglycemia and the pancreatic lesions observed after the administration of hydrocortisone were similar to those reported in cortisone treated rabbits.[1-3,27,28] In the present study, growth hormone administered simultaneously with hydrocortisone appeared to produce a slightly more severe diabetes than that induced by the steroid alone. This is in agreement with the report that simultaneous administration of the two hormones resulted in an accentuation of the diabetes.[22] The increased severity was accompanied by some accentuation of B cell degranulation and increased glycogen deposit in pancreatic duct epithelium and in B cells. It was not, however, possible to produce marked B cell vacuolization and no evidence of destruction of these elements was found. Growth hormone did not prevent the development of weight loss in the rabbit and many animals receiving this substance died of infection. These findings are contrary to previous reports that somatotropin administered simultaneously with cortisone prevents both the weight loss and increased incidence of infection which is found ordinarily in steroid-treated rats.[29]

In the present experiments intra-islet dilatation of ductules was frequently observed. This lesion has been interpreted by some as indicative of cyst formation within islet tissue.[30] It seems, however, to be related to ductular epithelial proliferation which occurs during treatment with steroids and appears to be similar to the alterations observed in the pancreas following ligation of its major duct.[3] ACTH administration caused mild diabetes and degranulation of the B cells. This would indicate that the rabbit adrenal is capable of secreting sufficient steroid to produce these abnormalities, a conclusion at variance with our own previous findings that ACTH administered to rabbits does not result in the prolonged hyperglycemic response to glucagon ordinarily observed after pretreatment with cortisone.[31] On the other hand, much smaller amounts of ACTH with shorter action were used in the earlier work. In support of observations in the present study, other authors have reported the appearance of B cell degranulation after the administration of ACTH to the rat but were unable to produce hydropic degeneration.[32] Contrary to previous reports, islet tissue did not appear to be damaged.[33] The metamorphosis of exocrine acinar cells[32] and of duct epithelium into B cells has been reported after ACTH treatment.[34,35] In the present study, although evidence suggestive of this type of transformation was seen in the form of continuity between duct and islet cells, no clear development of B cells could be demonstrated.

In our animals B cell degranulation could be identified unequivocally only 48 hours after beginning the administration of hydrocortisone with or without growth hormone. A similar timing has been observed in the rabbit after treatment with cortisone[2] and after the administration of orinase.[36] The degranulation which occurs in the rat pancreas after the intracardiac administration of glucose requires only 15 minutes.[37]

The results in the present study are in agreement with previous findings that during cortisone-induced diabetes in rabbits,[1,2] growth hormone-induced diabetes in dogs, as well as cortisone and/or growth hormone-treated dogs which have been partially pancreatectomized,[38-40] duct glycogen deposition precedes that appearing in B cells and the amount of glycogen increases concomitantly with the severity and duration of the diabetes. This observation has led to the hypothesis that hydropic degeneration is not due to functional exhaustion of B cells but is a facet of the increased cellular glycogen deposit found in diabetes, as is that observed in the kidney and myocardium. The reason for this predilective deposition of glycogen in specific sites has been an unsettled problem. Its appearance in pancreatic duct epithelium could be explained on the presumption of an increased concentration of glucose in the external pancreatic secretion in diabetes and its absorption from the lumen by the duct epithelium.[2,38,39] The fact that it appears more readily in B cells than in acinar elements might be due to the greater vascularity of islet tissue. In support of this concept, it was noted in the present experiments that glycogen appeared first in the B cells at their vascular poles (Fig. 2). The question of why glycogen is not found in A cells or in D cells is still unanswered. Furthermore, proliferating ductular epithelium usually does not contain glycogen.[1] This would lead to the conclusion that B cells and resting duct epithelial cells have some common metabolic denominator which permits the accumulation of glycogen within the cell.

One of the main arguments against accepting the occurrence of glycogen deposition as a passive sequela of hyperglycemia is the experiment which has shown that, in the alloxan diabetic animal, the administration of insulin in dosages insufficient to reduce hyperglycemia significantly prevents glycogen deposition.[41] This experiment was thought to prove that hypo-insulinemia was the cause of the intracellular deposit. However, it has also been demonstrated that the infusion of glucose directly into the pancreatic artery resulted in hydropic degeneration concomitant with peripheral hypoglycemia.[42] The

hypoglycemia appeared to indicate increased insulin output at the time that hydropic changes were occurring. In the present study it can be assumed that rabbits treated with steroids or growth hormone have adequate or even increased insulin output.[2,43] Nevertheless, they still manifest glycogen deposition. This would seem to negate the conclusions drawn in the experiments mentioned above[41] and suggest that the hyperglycemia *per se* is responsible for glycogenization.

Summary

A study has been conducted on the effect of hydrocortisone, hydrocortisone and growth hormone, and adrenocorticotropic hormone on the histologic structure of the pancreas and on the level of the blood sugar in the rabbit. The greatest degree of hyperglycemia was obtained after the simultaneous administration of hydrocortisone and growth hormone. The lesions in the pancreas consisted of B cell degranulation and glycogenization of duct epithelium and islet B cells. The lesions became more pronounced with increasing severity and duration of the diabetic state. Degranulation usually first became apparent after 2 days of treatment. Glycogen in ductular epithelium usually appeared by the 5th to 6th day and glycogenization of B cells was manifest at about 8 days. The earlier occurrence in ductular epithelium was in accord with previous findings. It was considered to indicate that the lesion was not a result of functional exhaustion of B cells but rather was due to hyperglycemia and was similar to the glycogenization observed in the kidney and myocardium in diabetes. Proliferation of pancreatic ductular epithelium also occurred in all groups of animals and seemed to be related to the duration of treatment rather than the degree of diabetes. Marked ductular epithelial proliferation within islets and cystic dilatation of ductules were noted. However, there was no unequivocal evidence of the formation of new B cells from ductular epithelium. It was found, contrary to other reports, that growth hormone administered simultaneously with hydrocortisone did not prevent the development of spontaneous infection in the rabbit.

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[*Illustrations follow*]

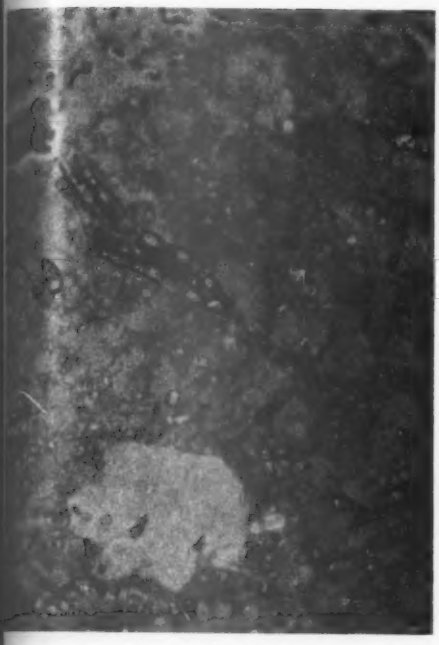
LEGENDS FOR FIGURES

FIG. 1. Pancreas of rabbit treated with hydrocortisone for 7 days, illustrating the presence of glycogen (black) in duct epithelium and none in B cells. PAS-hematoxylin stain. × 375.

FIG. 2. Pancreas of rabbit treated with hydrocortisone for 8 days, showing earliest appearance of glycogen (black granules) in pericapillary regions of islet (arrows). Glycogen is also seen in ductular epithelium (D). PAS-hematoxylin stain. × 780.

FIG. 3. High power view of pancreas of rabbit treated with hydrocortisone for 21 days, illustrating ductular epithelial proliferation (D) within islet, in close contiguity with B cells (B) and A cells (A). Aldehyde-fuchsin trichrome stain. × 780.

FIG. 4. Pancreas of rabbit treated with hydrocortisone for 14 days, illustrating intra-islet ductules (arrows) containing secretions. Aldehyde-fuchsin trichrome stain. × 375.



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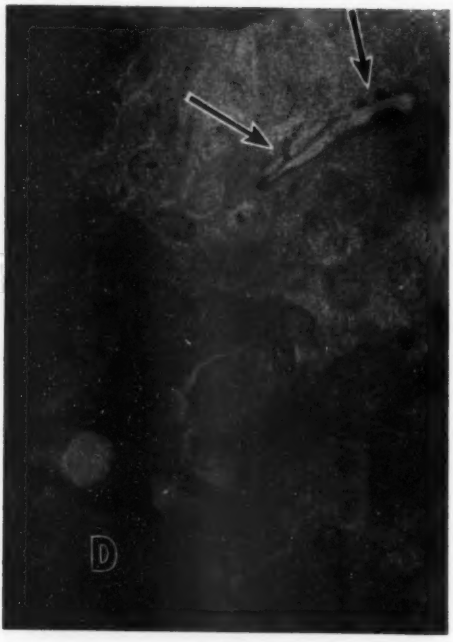

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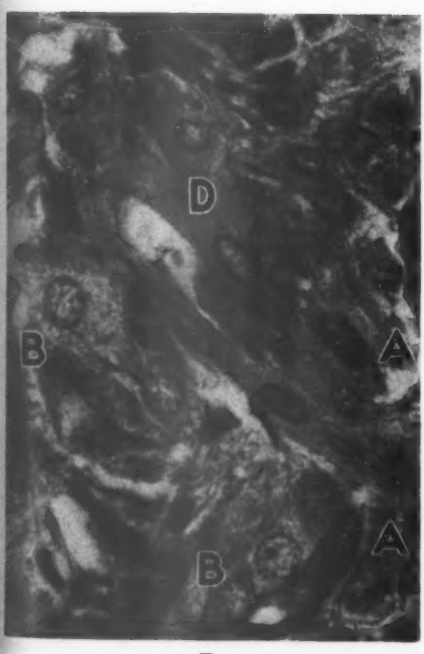

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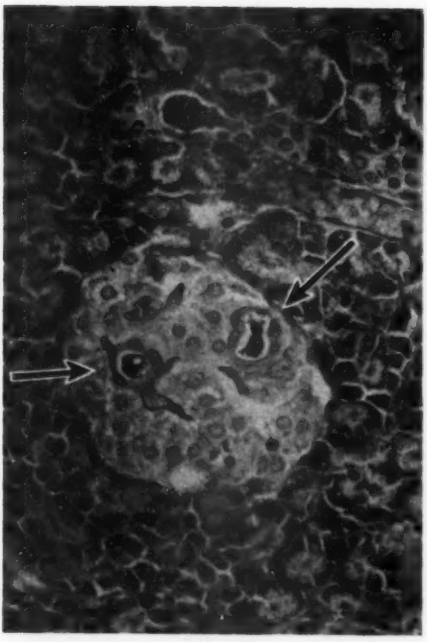

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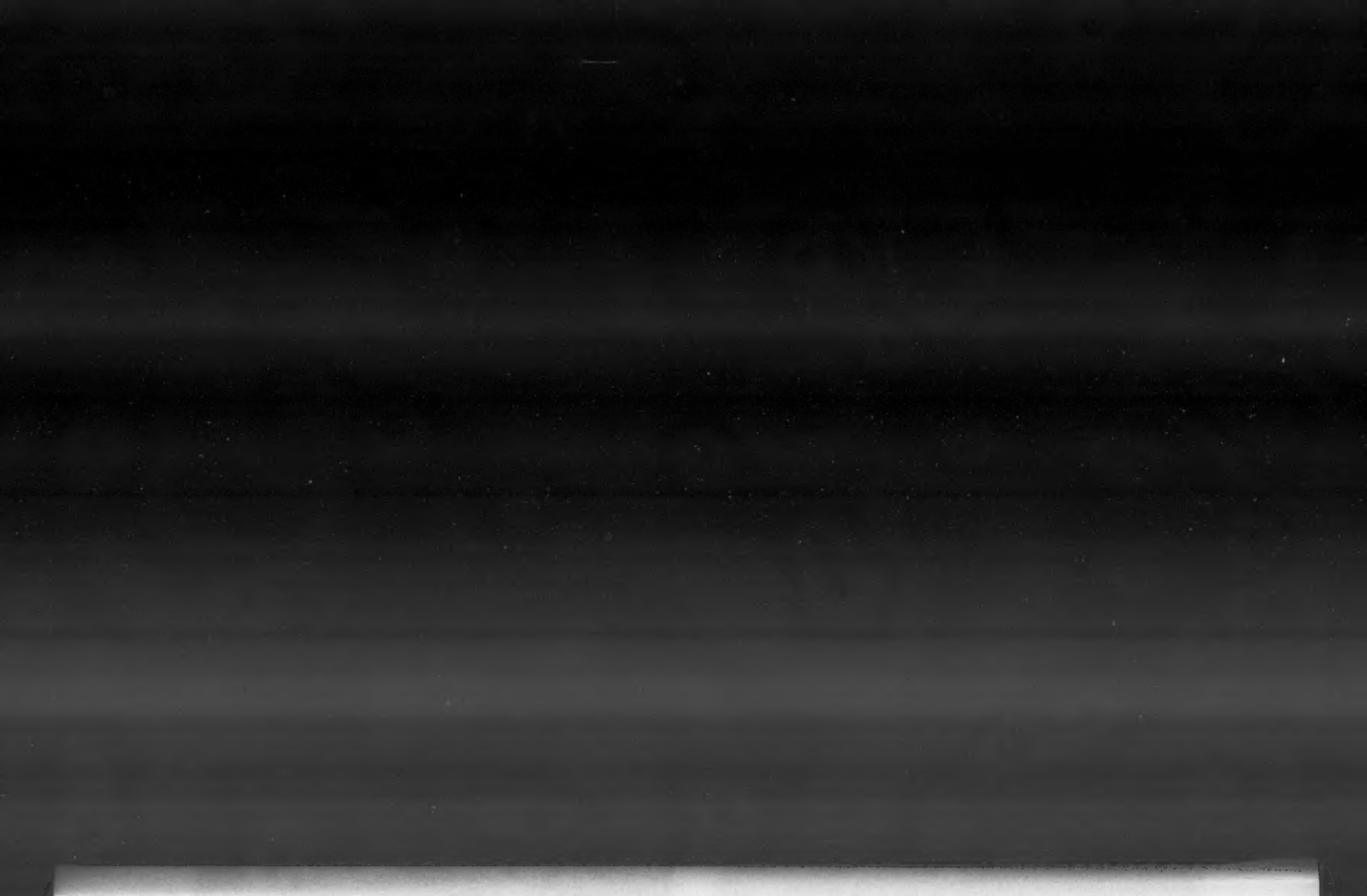
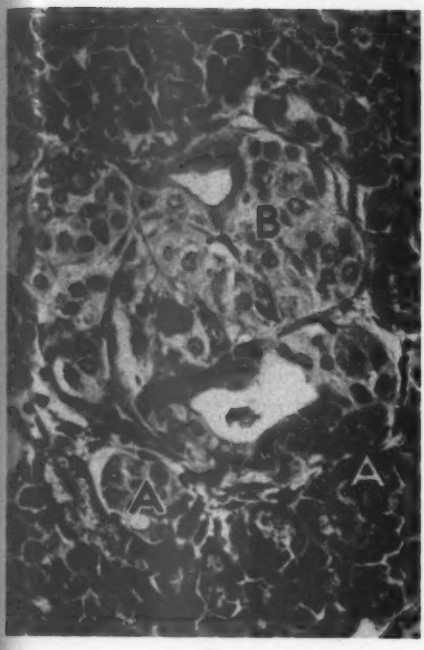
FIG. 5. Pancreas of rabbit treated with hydrocortisone for 21 days, illustrating proliferation and dilatation of ductules within islets. The ductular epithelium is thinned out in places and cannot be recognized. The B cells (B) are degranulated and pale. The A cells (A) are black. Aldehyde-fuchsin trichrome stain. × 375.

FIG. 6. Pancreas of rabbit treated with hydrocortisone for 21 days, illustrating an islet with a cystic space apparently lined by degranulated B cells. Aldehyde-fuchsin trichrome stain. × 375.

FIG. 7. Pancreas of rabbit treated with hydrocortisone for 19 days, illustrating pancreatic lobules (arrows) composed of dilated ductules and de-differentiated acinar tissue (arrows). Modification of Masson's trichrome stain. × 95.

FIG. 8. High power field from section shown in Fig. 8, illustrating the proliferation of ductular epithelium and dilatation of lumens in a comparatively intact islet. The A cells appear black. Modification of Masson's trichrome stain. × 280.





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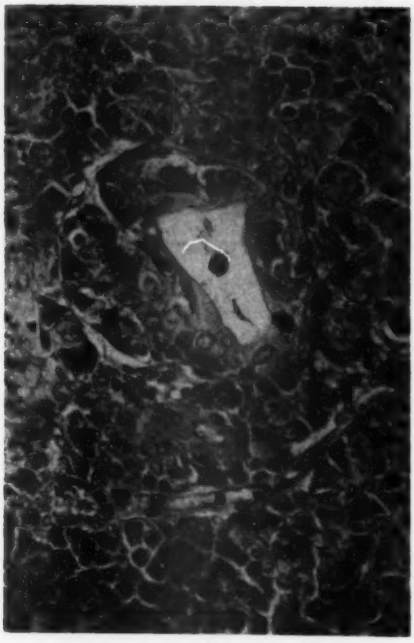

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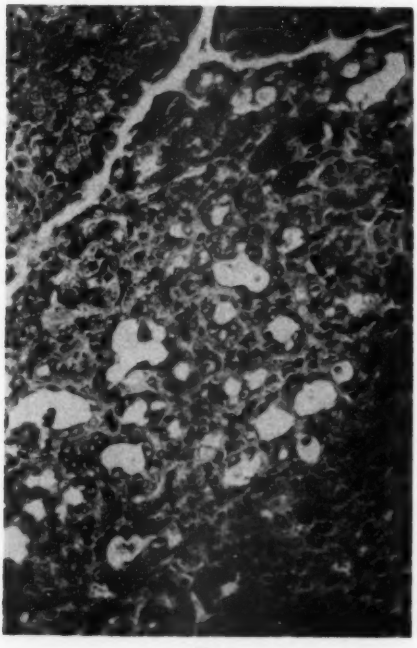

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ADRENAL CHANGES IN POST-TERM INFANTS AND THE PLACENTAL DYSFUNCTION SYNDROME*

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Prolonged gestation, and its effects upon the fetus, has been a subject of long-standing controversy. This is due in part to the difficulty of calculating accurately the length of gestation, and in part to lack of agreement as to what constitutes the so-called postmature infant, clinically or pathologically. Criteria for the diagnosis of postmaturity have been variably based on prolongation of gestation, and excessive fetal weight or length.[1] As a result, no agreement has been reached as to the relationship of postmaturity to perinatal mortality or morbidity.

Physiologic studies demonstrating a diminution in placental function near and after term have tended to corroborate the opinion of most investigators that prolonged gestation can be associated with deleterious effects on the fetus.[2-4] The placenta is known to stop growing before term and its permeability progressively diminishes after the 36th week of gestation. Functional reserve of the organ at this time is slight or absent. In pregnancy prolonged to 43 weeks, a significant diminution in oxygen content of the umbilical vein blood has been demonstrated. The work of Kloosterman and Huidekoper[5] is particularly enlightening in this regard. Two thousand placentas from consecutive births were studied and in each instance the placenta was characterized as to its fetal weight ratio and degree of infarction. From a statistical evaluation of the results, it became apparent that placental infarction, while having little influence on placental weight, was associated with a significant diminution in fetal weight. These changes were seen with greater frequency in gestations prolonged over 290 days. In two thirds of unexplained intra-uterine deaths, the placenta either was the seat of significant infarction or was hypoplastic.

Clifford in 1954[6] described a neonatal syndrome bearing a statistical relationship to prolonged gestation, which he termed "the placental dysfunction syndrome." In these cases, the newborn infant reveals evidence of recent weight loss, and small size for the calculated length of gestation. The skin may be dry, wrinkled, scaling and devoid of vernix caseosa. The presence of thick, yellowish meconium in the amniotic fluid results in a yellowish staining of the skin and umbilical cord. Since about 20 per cent of these infants have a calculated gesta-

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tion of 300+ days, the syndrome has been synonymously termed the "postmaturity syndrome," although it has been observed in term and preterm infants as well.[7] Mortality in Clifford's series of 37 infants was 16 per cent and morbidity, 46 per cent. The features of this syndrome are consistent with the effects of anoxia and malnutrition, which in all probability result from placental insufficiency.

Pathologic studies have been few and unproductive of evidence by which either post-term infants or those with the placental dysfunction syndrome could be characterized morphologically. The purpose of this study is to explore the significance of changes observed in the fetal adrenal cortex of infants, clinically considered to be post-term or suffering from the placental dysfunction syndrome.

Materials and Methods

The necropsy records of the Institute of Pathology of Western Reserve University from 1952 to date were examined. Stillborn infants dying within 36 hours of delivery and newborn infants dying before 3 days of life, with the clinical diagnosis of prolonged gestation, postmaturity, or the placental dysfunction syndrome, were selected for study (Fig. 1). Infants showing maceration or tissue autolysis were excluded. Criteria for postmaturity were based solely on calculated age of gestation and included cases of 41 weeks or more gestation. The clinical diagnosis of the placental dysfunction syndrome was based on Clifford's criteria.[6] Eleven cases were thus found and an additional case was provided by Dr. A. J. Segal of St. Luke's Hospital, Cleveland.

The adrenal glands, which had been fixed in 10 per cent formalin, paraffin-embedded, and stained with hematoxylin and eosin in the usual manner, were examined and evaluated. Sudan IV stains were performed on frozen sections in some of the cases. The pertinent clinical and pathologic data are recorded in Table I. The adrenals from 138 consecutive perinatal necropsies were studied in a similar fashion for statistical comparison.

Results

General Pathologic Observations

Most of the babies died with massive atelectasis of the primary type commonly seen in neonates. In addition, there were widespread passive hyperemia and focal visceral hemorrhage interpreted as consistent with the effects of anoxia. In 3 of the cases additional lesions contributing to death were acute pneumonitis (cases 2 and 6) and subarachnoid hemorrhage (case 11). In cases 7 and 12 there was evidence of cord

TABLE I

Summation of Important Features of 4 Infants with Placental Dysfunction Syndrome and 8 Post-term Infants

Case	Gestation (weeks)	Died at	Weight (gms.)	Length (cm.)	Placental Weight (gms.)	Placental character	Placental coefficient*	Clinical diagnosis	Necropsy	Wt.	% F. C.†	Category of lesion
1	41	28 hr.	1900	44	380	Small, no infarcts	.20	Placental dysfunction syndrome	Atelectasis neonatorum Wide-spread hyperemia	4.5	68%	B
2	40	28 hr.	1820	43	330	4+ infarction	.18	Caesarean section Placental dysfunction	Focal pneumonitis with hyaline membranes	1.8	50%	C
3	40	60 hr.	1890	43.5		Small, fibrotic		Placental dysfunction	Atelectasis neonatorum Wide-spread hyperemia	2.5	70%	A
4	36	31 hr.	1700	44		Small, fibrotic		Prematurity Placental dysfunction	Atelectasis neonatorum Widespread visceral hyperemia	2.0	60%	B
5	43	SB	3840	54	420	Focal calcification	.108	Post-term delivery SB	Atelectasis neonatorum Passive hyperemia	8.5	80%	B
6	43	SB	2570	49				Postmaturity	Acute pneumonitis Visceral hyperemia	9.5	70%	B
7	44	SB	3350	53				Intra-uterine death. Post-term. Cord around foot	Atelectasis neonatorum Visceral hyperemia	10.0	60%	A
8	41	1 day	4250	55				Postmaturity Caesarean section	Atelectasis neonatorum Visceral hyperemia	11.7	70%	B
9	43	SB	3200	51	400	No infarction	.125	Post-term infant SB	Atelectasis neonatorum Focal pulmonary hemorrhage	7.5	85%	B
10	42	1 day	2510	52				Neonatal atelectasis Post-term birth	Atelectasis neonatorum	3.8	60%	B
11	43	3 days	3930	51				Postmaturity	Recent subarachnoid hemorrhage Petechiae of lungs, heart, liver	8.5	75%	B
12	42	SB	2860	48	435	4+ infarction and calcification	.152	Postmaturity Cord around neck Pre-eclampsia	Atelectasis neonatorum Wide-spread hyperemia	11.0	80%	B

* Placental co-efficient = $\frac{\text{Placental weight (gms.)}}{\text{Fetal weight (gms.)}}$.

† % F. C. — Fetal cortex thickness as % of total cortex.

SB — Stillborn.

compression, and in the latter case, maternal pre-eclampsia was also present. Thus, in 3 of the 4 cases of placental dysfunction, lethal lesions other than those attributable to anoxia were not present, nor were they present in 4 of 8 post-term infants. Other organs were not remarkable, particular attention being paid to the endocrine glands. In the few cases in which the placenta was examined and weighed, 2 of the 4 in the placental dysfunction group showed a significant increase in placental coefficient according to Kloosterman's standards,[5] while in the post-term infants, only one of 3 showed a significant increase.

Adrenal Changes

Three categories of adrenal lesions were observed:

A. Diffuse Vacuolar Degeneration and Hyperemia of the Fetal Cortex. The sinusoids were markedly engorged with blood. Focal hemorrhages were sometimes present in the perimedullary portion of the fetal cortex. The fetal cortical cells showed marked vacuolation and Sudan IV stains demonstrated relative lipid depletion. The adult cortex was of normal thickness and the definition of the zona glomerulosa and fasciculata was not clear. The fetal cortex constituted approximately 80 per cent of the total cortical thickness. The less severe varieties of this change were interpreted as being within the range of normal. Cases 3 and 7 were thus considered to represent merely variations of the normal (Figs. 2 and 3).

B. Diffuse, Hemorrhagic Necrosis of the Fetal Adrenal Cortex. The fetal zone was partially or completely replaced by recent hemorrhage. Residual cells showed vacuolar degeneration, while most of the cells in the hemorrhagic area were necrotic. In the less affected peripheral portions, intense hyperemia and less severe vacuolar degeneration were present. In all instances the definitive cortex appeared intact and of relatively normal thickness. A variable degree of differentiation of the adult cortex into two zones was present. The fetal cortex made up about 80 per cent of the total cortical thickness. This change was the one most commonly observed in this series, occurring in cases 1, 4 to 6, and 8 to 12 (Figs. 4-6).

C. Preponderance of Adult Cortex. This change was observed in only one patient (case 2, Fig. 7). The adrenals were small, weighing 1.8 gm. The adult cortex was thicker than normal and well differentiated into a zona glomerulosa and fasciculata. The fetal cortex was narrowed to less than 50 per cent of the total cortical thickness and showed no evidences of active degeneration. A thin fibrous band was present around the medullary elements which were quite prominent. Table I represents a summation of these findings.

Significance Test

In this series of 8 post-term infants and 4 infants showing the placental dysfunction syndrome, all but 2 showed significant lesions of the fetal adrenal cortex. To test the significance of these findings, 138 consecutive necropsies of perinatal deaths, including stillbirths and infants less than 3 days old, were studied. Autolyzed fetuses, post-term infants and infants over 3 days of age were excluded. Adrenal lesions of the severity of category B or C were present in 24.6 per cent of this control group and in 83.5 per cent of the study group. The data from the control cases are presented in Table II. The difference in incidence is significant, the p value being less than 0.01.

TABLE II

Analysis of 138 Consecutive Perinatal Deaths

	Premature infants		Term infants		Premature and term infants	
	Adrenal lesions	Total number	Adrenal lesions	Total number	Adrenal lesions	Total number
Abruptio placentae	4	14	1	3	5	17
Birth trauma and breech delivery	3	4	0	2	3	6
Cord compression	0	6	0	1	0	7
Toxemias of pregnancy	4	6	0	1	4	7
Erythroblastosis fetalis	2	6	1	5	3	11
All others including congenital anomalies	14	73	5	17	19	90
Total	27	109	7	29	34	138
% of cases with adrenal lesions	24.8%		24%		24.6%	

In two of the cases reported there was cord compression and in another, maternal pre-eclampsia. To determine how these and other potentially anoxic conditions might be related to adrenal lesions, the 138 control cases were divided into sub-groups including abruptio placentae, birth trauma and breech extraction, cord compression, toxemia of pregnancy, and erythroblastosis fetalis, for both term and preterm infants. The incidence of adrenal lesions was essentially the same in the term and premature sub-groups. Although the figures are too small for statistical evaluation, no correlative trends were outstanding in these anoxic conditions, except possibly in birth trauma and toxemia.

DISCUSSION

Reviewing the morphologic features of this group of post-term infants and those with the placental dysfunction syndrome, nothing was observed which could be construed as pathognomonic of either condi-

tion. Three of the 5 placentas examined in this series showed a striking elevation in the placental coefficient, but no conclusions are warranted with so few examples. Degenerative and hemorrhagic lesions of the fetal adrenal cortex occurred with significantly greater frequency in the study group than in a random population of perinatal necropsies. The changes observed in the adrenals are qualitatively indistinguishable from normal involution of the fetal adrenal cortex, which typically does not become prominent until after the third day of life[9-13] with hemorrhage and degeneration of the zone reaching a peak during the second week of life. It is thus possible that these changes might represent precocious involution having its inception *in utero*, since all these infants were less than 3 days old. The assumption that this is precocious involution is subject to the criticism that the range of normal variation of the process is not well understood. Benner[10] described 5 instances of apparently precocious involution in a series of 33 newborn and stillborn infants to which she could not ascribe any significance. The dysgenesis of the adrenals in anencephalic monsters has been attributed to precocious involution of the fetal cortex,[12-14] and the adrenals of case 2 were reminiscent of those seen in anencephalia.

The hemorrhagic character of many of the lesions suggests the possibility that these lesions could be traumatic or anoxic in origin. Hemorrhage into neonatal adrenals has been observed in association with birth trauma, usually in breech extractions[15,16] and was deemed capable of producing fatal acute adrenal insufficiency.[17,18] In this series, trauma and breech extraction were not present. Anoxia cannot be excluded as a causative factor. Most infants, however, dying with severe anoxic hemorrhages in other viscera, do not show comparable adrenal hemorrhage. Furthermore, in analyzing perinatal conditions typically associated with fetal anoxia, no striking relationship to adrenal change was apparent in any one.

The physiologic factors concerned with maintenance and involution of the fetal adrenal cortex are not well understood.[12,13] There is evidence suggesting that the placenta is of importance, either as a result of the gonadotropins it produces, or the maternal hormones it may transmit.[12-14] It is thus possible that the changes observed in the fetal adrenal cortex in post-term infants and in those with the placental dysfunction syndrome, reflect inadequacy of placental function, whether this be transfer or secretion. In the absence of certain knowledge concerning the function of the fetal zone,[12,13] to suggest that the lesions might be lethal by contributing to adrenal insufficiency would be purely speculative.

Summary and Conclusions

1. Twelve newborn and stillborn infants, with the placental dysfunction syndrome or considered to be post-term were studied pathologically, particular attention being paid to the adrenal glands.

2. Hemorrhagic and degenerative lesions of the fetal adrenal cortex were observed to occur in the study group with significantly greater frequency than in a random sample of perinatal necropsies.

3. Since these infants were all less than 3 days of age, it is possible that these changes might represent precocious involution of the fetal adrenal cortex having its inception *in utero*.

4. It is suggested that these changes may reflect inadequacies of placental function.

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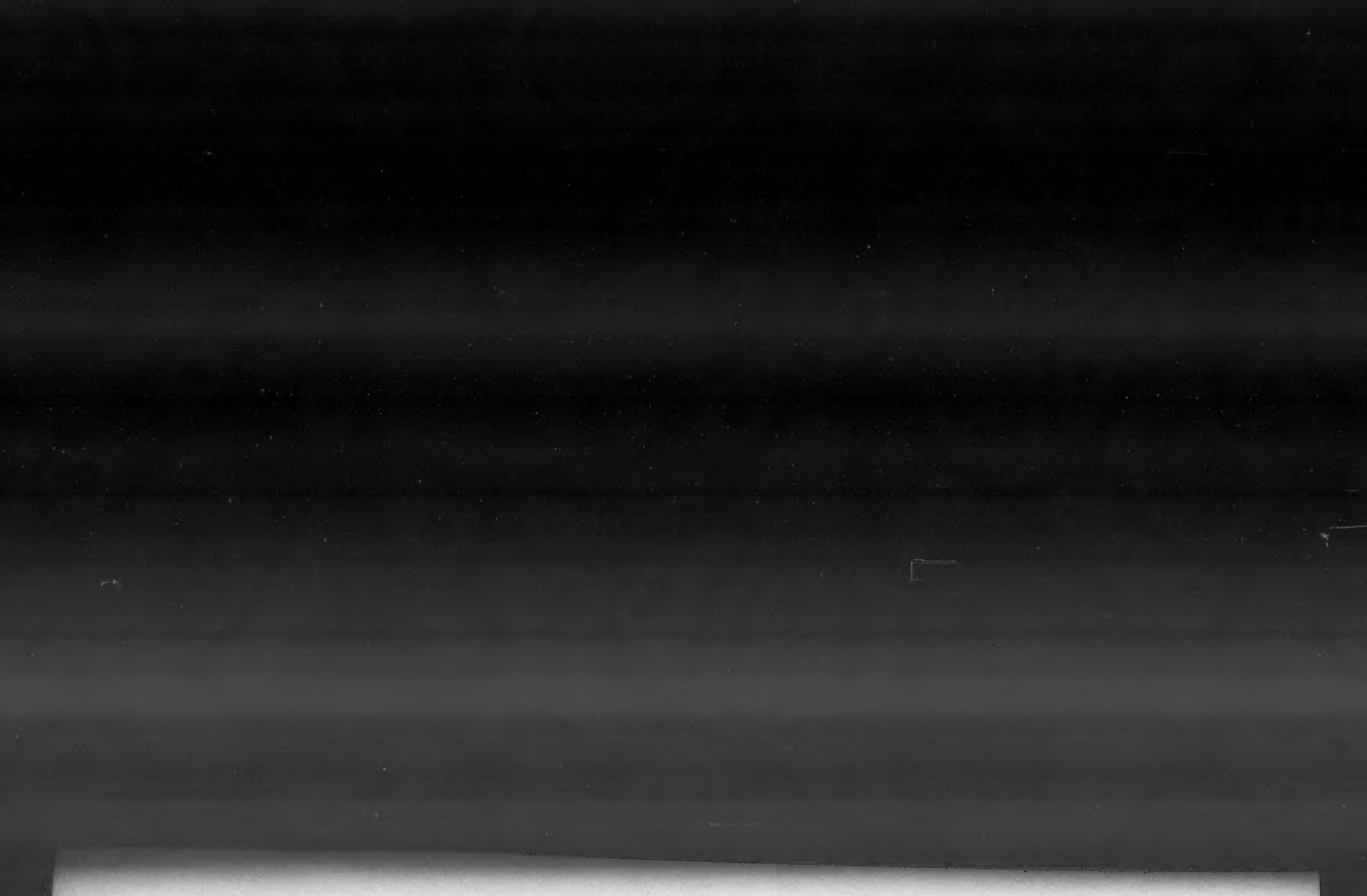
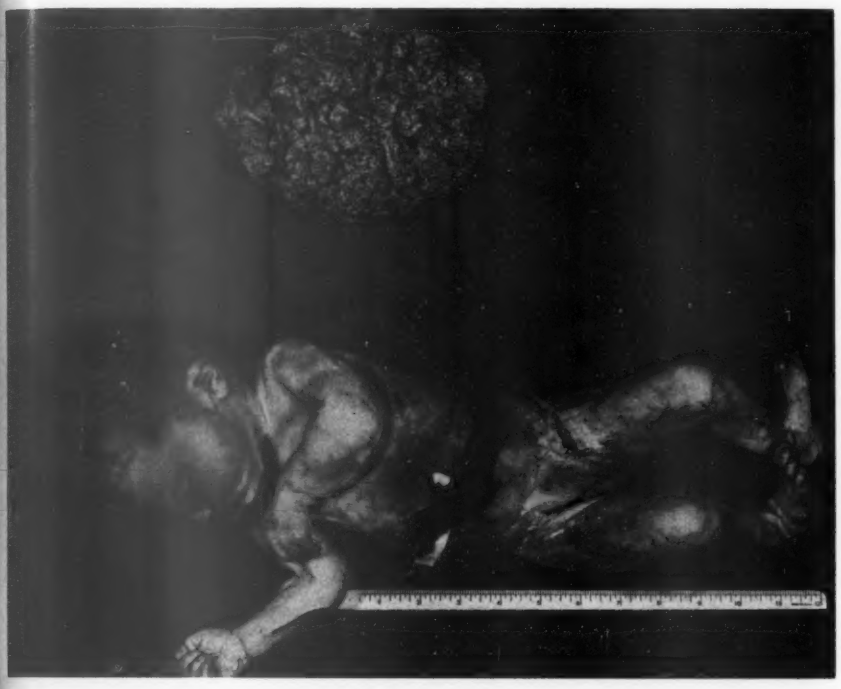
LEGENDS FOR FIGURES

FIG. 1. An example of the placental dysfunction syndrome. The infant was stillborn following 43 weeks gestation. The skin is dry, wrinkled, and stained by meconium. The placenta is disproportionately small and fibrotic.

FIG. 2. Representative cut surfaces of adrenal glands. "A" is from a stillborn term infant and "B" is from a stillborn premature infant. Note the relative cortical thickness and absence of hemorrhagic change. "C" is the adrenal from case 5, stillborn after 43 weeks gestation. Note the hemorrhagic change of the central fetal cortex, with the peripheral pale halo representing intact adult cortex. "D" is the adrenal of a post-term infant (case 9) showing a narrower central zone of hemorrhage. "E" is from case 3, an infant with placental dysfunction syndrome; the hemorrhagic zone is thin and less intense. "F" is the hypoplastic adrenal of case 2, also a patient with placental dysfunction syndrome. Hemorrhage is not present, but the marked thinning of the entire cortex is apparent.

FIG. 3. A microscopic section of the adrenal cortex of case 3. The majority of the fetal cortex is intact. Centrally, there is marked hyperemia and minimal vacuolar degeneration of cells. This severity of change was not considered a significant deviation from normal and is referred to as category A. AC—Adult cortex. FC—Fetal cortex. Hematoxylin and eosin stain. × 86.





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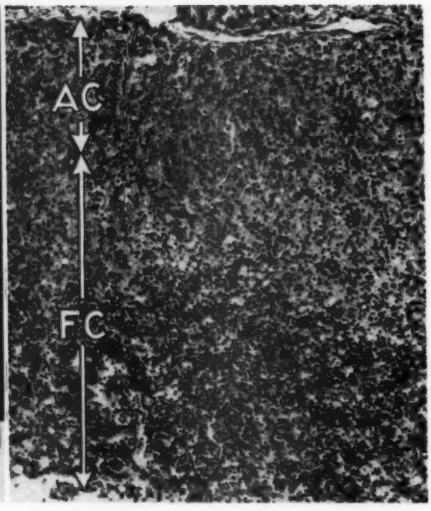

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FIG. 4. Section of the adrenal gland of case 9. A central zone of hemorrhage is present in the fetal cortex in association with beginning cellular degeneration, characterized by cytoplasmic swelling and vacuolation. The adult cortex is thin and not remarkable. Category B. Hematoxylin and eosin stain. × 86.

FIG. 5. Section of the adrenal of case 11. The fetal cortex shows an extreme degree of vacuolar degeneration and focal hemorrhage. The adult cortex is thin and not remarkable. Category B. Hematoxylin and eosin stain. × 86.

FIG. 6. Section of the adrenal from case 5. The fetal cortex is the seat of extensive hemorrhagic necrosis. The adult cortex is thin and intact. Category B. Hematoxylin and eosin stain. × 86.

FIG. 7. The adrenal from case 2. The entire cortex is thin, and the adult cortex occupies about one half the total thickness. The latter structure shows a well-defined zona glomerulosa and zona fasciculata. The fetal cortex is markedly attenuated, and degenerative changes are not prominent. Category C. Hematoxylin and eosin stain. × 86.

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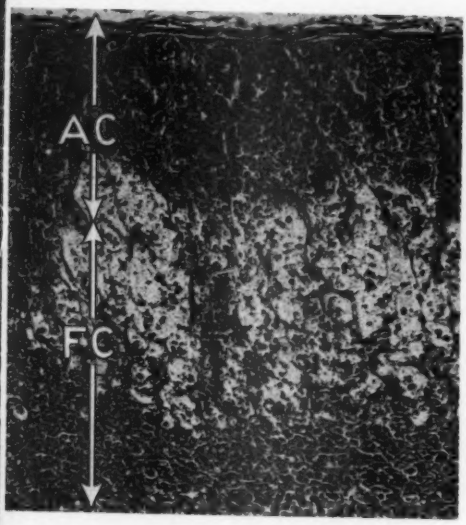

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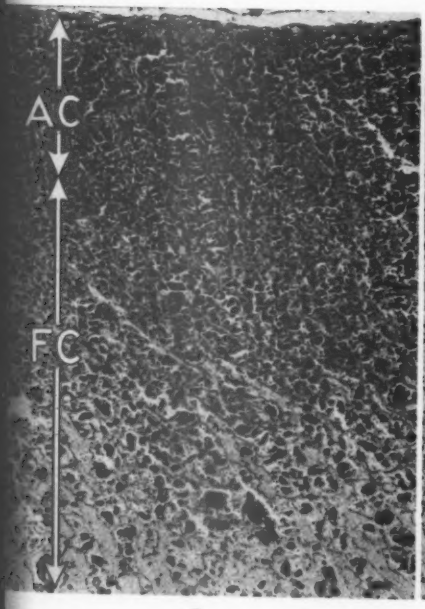

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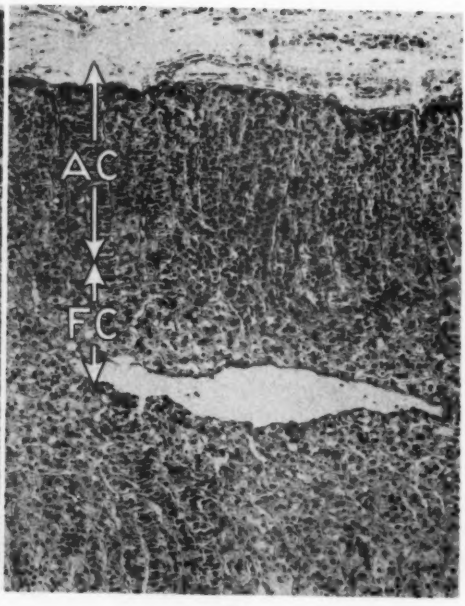

7

CARDIAC HYPERTROPHY AS A MANIFESTATION OF CHRONIC ANEMIA *

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Occasionally it is necessary to evaluate the role that anemia may play in the production of hypertrophy of the heart. This question may be raised when enlargement of the heart is encountered and is unaccompanied by valvular lesions or other known causes of cardiomegaly but appears in patients with anemia of significant degree. Too often, in the literature on this subject, the role of factors other than anemia is not or cannot be evaluated, and the opinions expressed are of little worth. The contribution of severe, chronic, uncomplicated anemia to the development of cardiac hypertrophy has never been conclusively demonstrated.

The occurrence of cardiomegaly in patients with severe chronic anemia has long been recognized. One of the first to note its presence was Irvine[1] who stated in 1877 that the most important change in the circulatory system in anemia was ". . . dilatation of the ventricles of the heart, especially, perhaps, of the left." In a report of two cases of chlorosis Hersman[2] noted that enlargement of the heart was commonly observed in this disorder.

With the advent of roentgenography it became possible to study variations in heart size with a higher degree of accuracy. Ball[3] was the first to report a case of cardiomegaly demonstrated radiographically in a patient with uncomplicated anemia in whom there was a return of heart size to normal following correction of the anemia. Since this time, a number of other authors[4-6] have shown that cardiac enlargement associated with chronic anemia may be reduced following successful treatment of the anemia. The roentgenogram is highly useful in the recognition of cardiomegaly, but its effectiveness in distinguishing between cardiac dilatation and hypertrophy is limited, as pointed out by Levine.[7] Although some writers[3,7,8] have attributed the cardiac enlargement in anemia to dilatation alone, others have believed that hypertrophy is present also.[4-6]

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Studies of necropsy specimens from patients with chronic anemia have not been conclusive. In some reports of instances of cardiac dilatation and hypertrophy associated with anemia, hypertrophy was shown to be present by necropsy examination,[9,10] but in one of these[10] the authors stated that they believed some factor other than the anemia must have been responsible for the hypertrophy. In the 12 cases of pernicious anemia reported by Goldstein and Boas[11] the average heart weight was 330 gm. This average exceeds by only 30 gm. the "normal"[12] weight of the heart. The heart weights ranged from 250 to 460 gm. The authors failed to state whether the anemia in their cases was accompanied by other disease states which might have resulted in cardiac hypertrophy. The authors concluded: "Cardiac dilatation, often accompanied by cardiac hypertrophy, is a common finding in patients with severe anemias." Cabot and Richardson[13] reviewed the necropsy reports on 19 cases of pernicious anemia. They reported that definite hypertrophy was present in 13 of these, slight hypertrophy in 4, and no hypertrophy in 2. Their results are somewhat invalidated by the fact that 4 of the 13 patients with definite cardiac hypertrophy had glomerulonephritis, and one of the remaining 9 had considerable valvular deformity. Among the 4 patients with slight hypertrophy one had glomerulonephritis.

Some experimental work has been reported in which the effect of chronic anemia on the size of the heart is described. Lüdke and Schüller[14] produced anemia in dogs and foxes by the use of venesection and by means of intravascular hemolysis. In their report they did not comment on the presence or absence of cardiac hypertrophy. They said that they found cardiac dilatation following the injection of hemolysin into the animals. Dilatation did not appear after the frequent withdrawal of small amounts of blood nor following the intravenous injection of distilled water.

Incidental to nutritional studies in which they were engaged, Forman and Daniels[15] found that rats with severe nutritional anemia had hearts weighing a great deal more than did those of nonanemic rats of comparable body weight. Similar investigations by Cowan,[16] who used both litter mate and size controls, confirmed the findings of Forman and Daniels that experimental nutritional anemia may be accompanied by cardiac hypertrophy. The possible relation of the nutritional deficiency to cardiac hypertrophy was not discussed in either of these papers. Although the composition of the various diets used in the experiments were stated, the authors did not say in what quantities the diets were given to the animals. Since the diets consisted of milk

with the addition of certain other substances, and since the niacin content of milk is low, one wonders whether the existence of beriberi with its accompanying heart disease was excluded in these rats.

In order to ascertain the effects of anemia alone on the heart, severe chronic anemia was produced in dogs by frequent, repeated venesections, and the hearts were studied at necropsy.

Methods and Material

Twelve young full-grown mongrel dogs were used in the experiment. Each of the animals was confined in a small mesh cage; none of the dogs were allowed to engage in any exercise, because of the difficulty in standardizing such exercise. Each dog was provided with one pound of Uncle Remus Hush Puppy Dog Feed daily. This is standard ration for dogs in our animal house.

Five of the dogs were designated as control animals. At monthly intervals for 5 months, 10 cc. of blood were withdrawn from each of these dogs for the determination of hemoglobin and serum protein values.

The 7 remaining dogs were designated as experimental animals. Three times weekly for a period of 5 months, blood was withdrawn from the femoral vessels of these animals. During the first 3 weeks of the experiment, sufficient blood was withdrawn to lower the hemoglobin level in the peripheral blood from an original value of approximately 17 gm. per hundred cc. to a level of approximately 5 gm. Following the initial lowering of the hemoglobin concentration, at each bleeding sufficient blood was withdrawn to maintain the hemoglobin level between 3.5 and 6 gm. An average of 175 cc. of blood was withdrawn from each of the experimental animals at each bleeding episode. Hemoglobin determinations were made on the blood from these dogs 3 times weekly. Serum protein determinations were made at monthly intervals.

It was intended to study chest roentgenograms of the dogs at regular intervals in order to determine whether any cardiac enlargement would be detectable by this means. Because of many technical difficulties encountered, these films were considered unsatisfactory for the purpose of determining the presence of cardiac enlargement.

Two of the experimental animals died in the course of the investigation shortly after venesections had been performed on them. The remaining 5 experimental animals and the 5 control animals were sacrificed 5 months after the initiation of the experiment. A complete necropsy was performed on each of the dogs. In none of the animals

were there peripheral edema or collections of fluid in the serosal cavities. Moderate congestion of the viscera was observed.

The hearts were examined in a uniform manner: the heart and lungs were removed *en bloc,* and the pulmonary vessels, the aorta, and the venae cava were then severed at the point at which they entered the heart. The hearts were opened, drained of blood, and weighed. They were then submitted to an experienced pathologist[17] who was asked to state whether, in his opinion, each of the hearts was hypertrophied. He was not told which of the hearts were from experimental animals and which were from control animals, and he was not told the weights of the hearts.

In order to determine the amount of tissue fluid, a small amount of myocardium was taken from an identical site in each of the 10 hearts. This tissue was weighed, frozen-dried, and reweighed. The percentage of water in each piece of tissue was thus determined.

Results

It is generally accepted that the existence of cardiac hypertrophy is best ascertained from direct inspection of the heart by an experienced pathologist. The results of this type of examination of the 10 hearts in our experiment are shown in Table I. This distribution of data has a probability of occurrence by chance alone of 0.008, and these results are statistically significant.

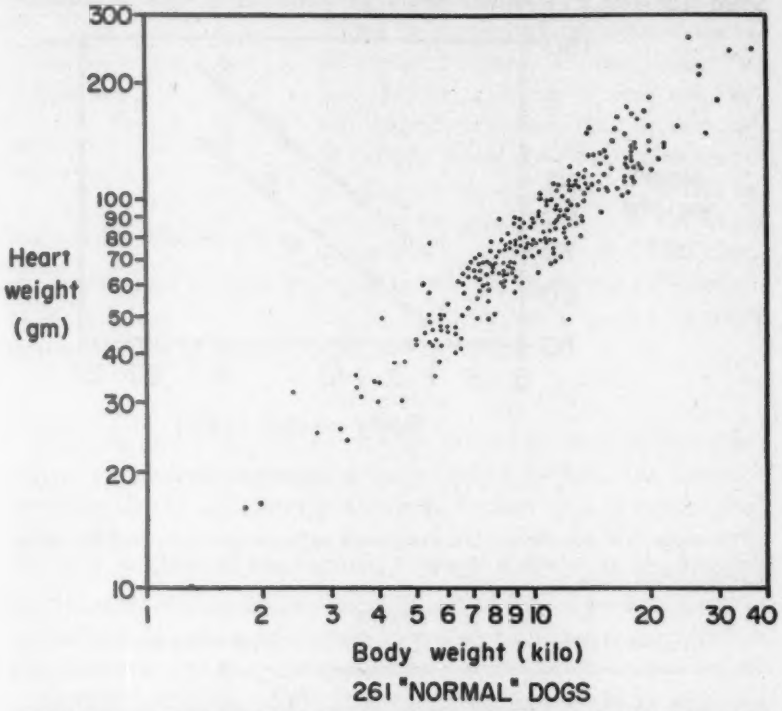
Table I

Evaluation of Cardiac Hypertrophy by Examination of the Gross Specimens

Hypertrophy	Experimental dogs	Control dogs
Present	5	0
Uncertain	0	1
Absent	0	4
	$p = 0.008$	

In Figure 1 are illustrated the hearts from 2 dogs, each of which weighed 12 kg. The heart on the left is from a control animal; it weighed 71.4 gm.; the heart on the right is from an experimental animal; it weighed 102.4 gm. In Figure 2 the left ventricular wall of an experimental dog (# 119) is seen to be much thicker than that of a control dog (# 155). Ventricular hypertrophy was present on the right side of the heart as well as on the left, and in all the experimental animals there was marked dilatation of both ventricles. Since all our animals were not of the same body weight as the 2 illustrated, a relationship between the heart weight and the body weight in normal dogs that would enable us to interpret our data was sought. In the literature[18-20] we found values for the heart weight and the body weight for 261 "normal" dogs. When

the logarithm of the body weight is plotted against the logarithm of the heart weight (Text-fig. 1) a straight-line relationship is seen to exist. Having established this relationship, we plotted the data from the ani-

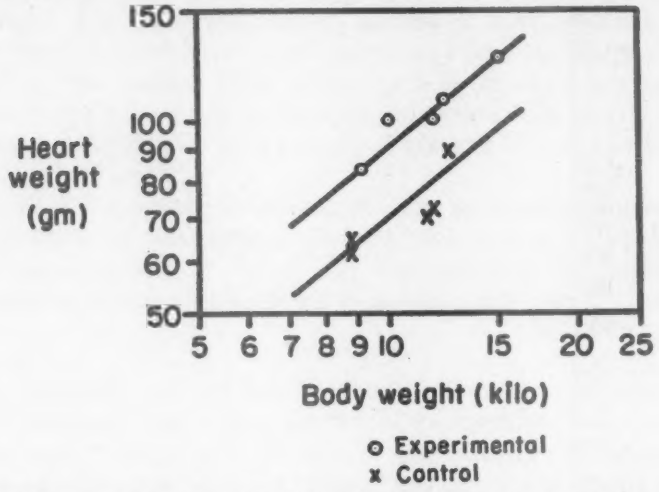


Text-figure 1. The logarithm of the body weight is plotted against the logarithm of the heart weight for 261 normal dogs, illustrating the straight-line relationship which exists between these two factors.

mals in our experiment in a similar way (Text-fig. 2). The covariant of these distributions for our experimental and control animals was determined in the standard fashion,[21] and it was found that this distribution of data is highly significant with $p << 0.001$ (Table II). This establishes the significance of the greater heart weight:body weight ratios seen in the experimental animals. In order to determine whether a diminished body weight was present in the experimental animals, thereby possibly affecting the ratio, the animals were weighed at the beginning and again at the termination of the investigation. It was found that none of the 10 animals had lost weight.

Edema of the hearts in the experimental animals, resulting from a hypoproteinemia induced by massive bleeding, was considered a possi-

ble factor in the weight increase. In none of the 10 dogs was there a significant decrease in the total serum protein or in the albumin fraction. This finding is in conformance with the reports of Whipple[22] who noted that with an adequate diet large quantities of protein could be



Text-figure 2. The logarithm of the body weight is plotted against the logarithm of the heart weight for the 10 dogs in the present experiment, and the trend lines of the two distributions are drawn.

TABLE II

Analysis of Covariant and Test of Significance of Adjusted Group Means

Source of variation	Degrees of freedom	Sums of squares and products			Errors of estimate		
		Sx^2	Sxy	Sy^2	Sum of squares	Degrees of freedom	Mean square
Total	9	0.0521	0.0520	0.0980	0.0460	8	
Groups	1	0.0040	0.0162	0.0657			
Within groups (error)	8	0.0481	0.0358	0.0323	0.0055	7	0.00079
For test of significance of adjusted means					0.0405	1	0.0405

$$F = \frac{0.0405}{0.00079} = 51.27 \quad p<<0.001$$

synthesized by the dog to replace that lost by bleeding. In order to ascertain whether an increased accumulation of fluid had occurred in the hearts of the bled animals, the percentage of water by weight was determined as described previously. In Table III it can be seen that the amount of water in the hearts of the 2 groups was almost identical.

The value of t for these two ranges of values is 0.397 which is not statistically significant.

The study of microscopic sections from the hearts of the control and experimental animals, as anticipated, helped to strengthen the other observations cited but were not conclusive. The detection of moderate or even considerable degrees of muscle fiber hypertrophy is difficult when no quantitative micrometric methods are applied. In this experiment the other methods of evaluation of cardiac hypertrophy gave such clear-cut results that micrometry was not thought necessary. The microscopic sections were of value in excluding such factors as fatty degeneration or inflammatory cellular infiltration.

TABLE III

Amount of Water in Hearts (per cent of total weight)

Experimental	Control
76 %	80 %
80	78
81	78
76	74
76	76
Mean=77.8%	Mean=77.2%

DISCUSSION

With the demonstration that anemia *per se* can cause hypertrophy, certain implications regarding therapy become evident. The necessity for correction of anemia in relieving the burden on a decompensated heart is obvious. Admittedly, the anemia is ordinarily not of primary concern. It is conceivable, however, in the light of the observations cited that on rare occasions severe chronic anemia alone may result in cardiac failure. On most occasions, anemia as a factor in congestive failure will be additive, and correctable.

Potential questions of great interest arise in connection with this study. One may analyze the relation between the amount and duration of anemia and the anatomic alterations in the heart. By varying the degree and duration of anemia in different series of dogs, one may discover something of the nature of the primary stimulus leading to cardiac hypertrophy. Some authors[5] believe that on a purely mechanical basis increased work load itself may be sufficient to cause hypertrophy. Others[2,3] have suggested that hypertrophy represents a reaction by the myocardium to some injury which it has sustained. With the uncomplicated experimental method we have at hand, direct investigation of such matters is possible and further investigation along this line is contemplated. Little attention has been given to the biologic phenomenon of hypertrophy of cells and organs. The demonstration that anemia may serve as a single factor in the production of cardiac hypertrophy may lead to further understanding of this phenomenon.

Summary

In order to study the effect of severe uncomplicated chronic anemia on the size of the heart, the authors produced anemia (3.5 to 6 gm. of hemoglobin per hundred cc. of blood) in dogs by frequent, repeated venesections. After 5 months the animals were sacrificed. The hearts of the experimental animals and those of normal control dogs were submitted to an experienced pathologist who found that a significant hypertrophy was present in the animals that had been bled. The ratios of the logarithm of the heart weight:logarithm of the body weight in the experimental and control groups were analyzed to determine the covariant, and it was found that a statistically significant ($p << 0.001$) difference existed between the two groups of ratios.

The possibility that edema was responsible for the difference in weights between the hearts in the two groups was excluded by the demonstration that the water content in the hearts did not significantly differ in the groups. The hypertrophy was found to involve both right and left ventricles and to be accompanied by dilatation of both chambers.

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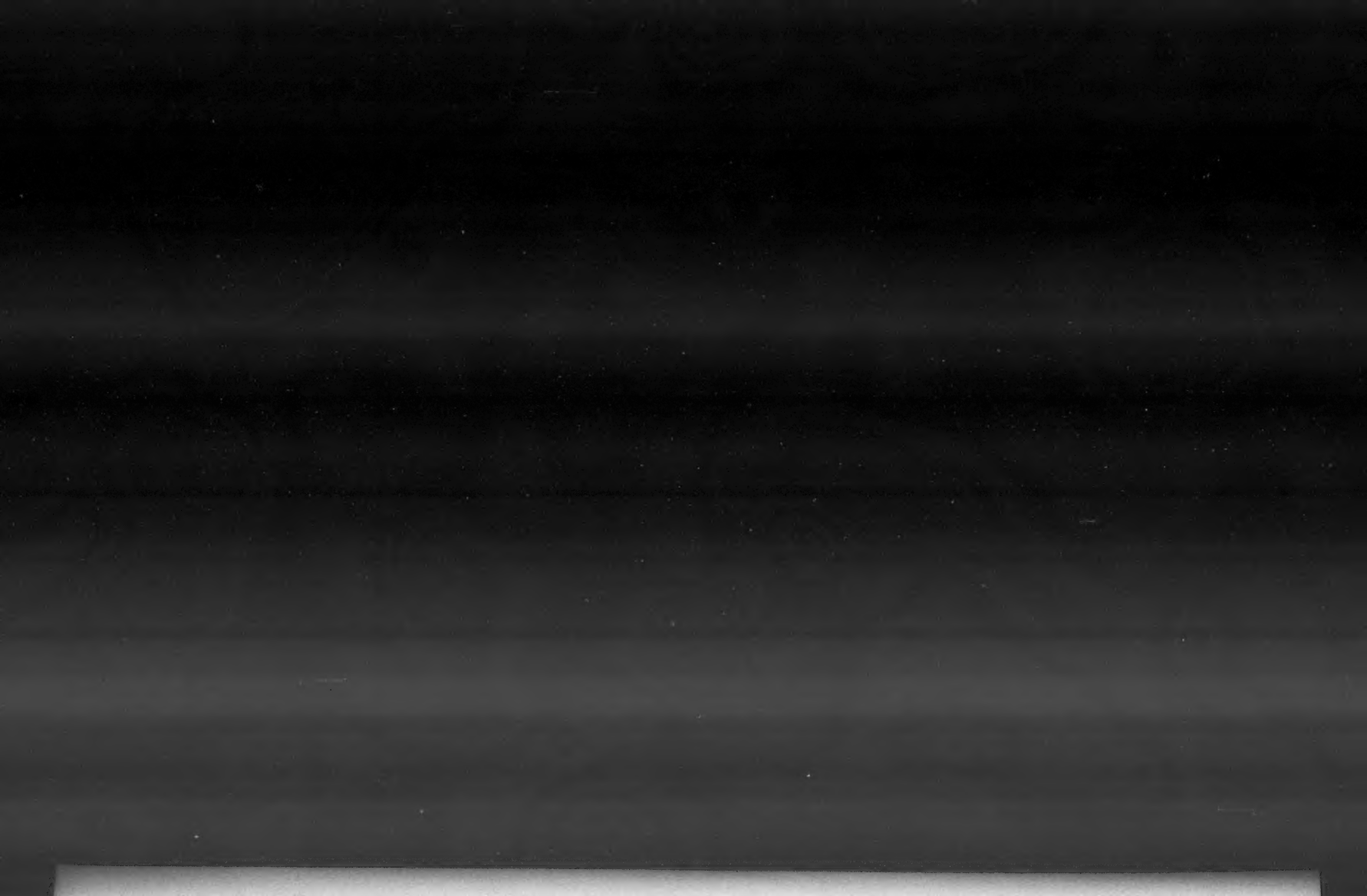
Grateful acknowledgment is given to Dr. John L. Shapiro, Professor of Pathology and Head of the Department, Vanderbilt University School of Medicine, for help in the formulation of this investigation and in interpreting the results; and to Dr. Margaret P. Martin, Associate Professor of Preventive Medicine and Public Health, for assistance in analysis of the data.

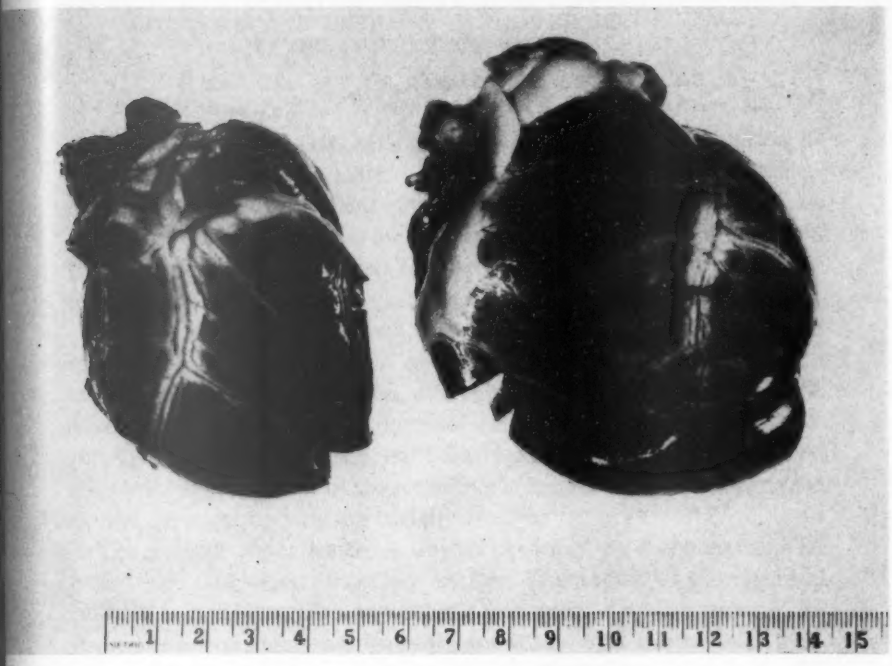
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Left. The heart from a 12 kg. dog (#155) in the control group. This heart weighed 71 gm.
Right. Heart from a 12 kg. dog (#119) in the experimental group. This heart weighed 102 gm.

FIG. 2. Tissue slices from identical places in the left ventricles of the two hearts shown in Figure 1. The slice on the right (from dog in the experimental group) illustrates the hypertrophy present in the myocardium.





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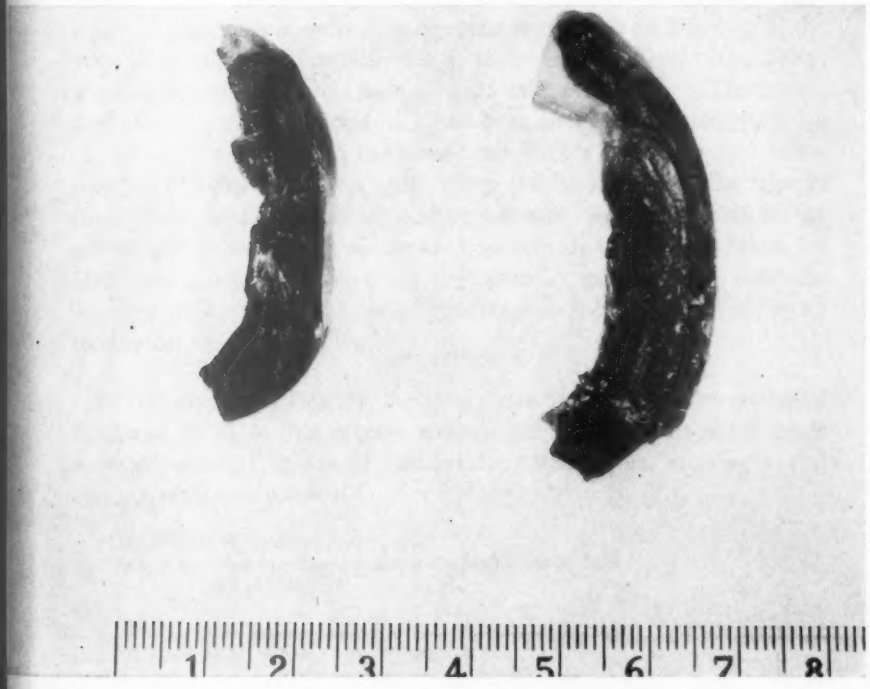

2

PULMONARY LESIONS IN MICE DUE TO FRAGMENTS OF HAIR, EPIDERMIS AND EXTRANEOUS MATTER ACCIDENTALLY INJECTED IN TOXICITY EXPERIMENTS*

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Earlier studies in this laboratory dealt with various problems of disease in laboratory animals.[1] Among other matters these were concerned with clinically silent and incidental lesions which might be mistaken for the effects of some experimental procedure. We suggested that the occurrence of gobbets of bone found in the lung of laboratory animals was the fortuitous result of aspiration of dust of pelleted diets.

The frequency of granuloma, vasculitis, and embolic lesions found in the lungs of mice makes it of signal worth to those who perform repeated intravenous injections, and subsequently study the pathologic effects on the lungs. Jaffé[2] does not mention such lesions as are depicted here. In our own case, until the "foreign material" was identified within the lesions, some of the latter might have been assumedly caused by the chemical undergoing toxicity studies.

The present paper concerns lesions produced by intravenously injected hair and other extraneous matter. The nature of the chemical injected is irrelevant. It was a soluble one, and the worker who administered the drug had had lengthy experience with intravenous techniques. There were 60 experimental mice, divided into 4 groups with 3 sets of 5 animals in each. The 4 groups were given an injection of the drug *via* a tail vein once daily for 5, 10, 15 and 20 days, respectively. Three different dose levels were given to each set of 5 mice. There were 4 groups of 5 control mice which had been similarly injected daily (5, 10, 15 and 20 days) with the same volume (0.3 cc.) of normal saline solution (Tables I and II). A 27-gauge needle was used. The mice in each group, and their corresponding controls, were sacrificed at the end of the 5, 10, 15 and 20 days. A variety of tissues was taken for histologic study, sections being prepared by conventional methods. The chemical compound injected produced no specific pathologic effect on any organ.

Observations

In our work with rodents, the total thoracic contents were generally fixed and sliced, so that in most sections, parts of all lobes of the lungs were represented; parts of the heart, main vessels, esophagus and trachea were also present.

* Received for publication, June 7, 1957.
† Now at the Wellcome Research Laboratories, Tuckahoe, N.Y.

The lungs of some of the first group of mice showed vascular, perivascular and focal lesions which raised the issue whether the compound had produced them. Included in these were very few lesions containing crystalline material, which we thought might have been precipitated out of the drug solution injected. Closer study revealed the presence of pulmonary granulomas and embolic lesions. Some of these contained foreign material, and what were manifestly, but mystifyingly, pieces of hair. Consequently, all lungs were examined in order to determine incidence and distribution of the various lesions. The results are summarized in Tables I and II. The numbers may surprise experimentalists who do repeated intravenous injections in mice.

TABLE I

Control Mice Injected with Normal Saline Solution

Group	No. of injections	Pulmonary granulomas	Granulomas with foreign bodies	Vasculitis	Vasculitis with foreign bodies	Endocardial lesions
I (5 mice)	5	0	0	0	0	0
II (5 mice)	10	1	0	20	6	0
III (5 mice)	15	2	1	21	6	1
IV (5 mice)	20	2	1	13	6	0
Total 20 mice		5	2	54	18	1

TABLE II

Mice Injected with Chemical Compound

Group	No. of injections	Pulmonary granulomas	Granulomas with foreign bodies	Vasculitis	Vasculitis with foreign bodies	Endocardial lesions
I (15 mice)	5	3	1	1	0	0
II (15 mice)	10	4	1	32	11	0
III (15 mice)	15	5	1	43	9	2
IV (15 mice)	20	5	1	27	7	2
Total 60 mice		17	4	103	27	4

There were focal granulomas (Fig. 1) composed of mononuclear phagocytes. These were scattered in random pattern throughout the lungs and could be picked out with a low scanning objective. All were not (in any one section) clearly related to a vessel. There were no necrosis, giant cells or hemorrhage. In some granulomas there were central crevices as if material had fallen out (Fig. 2). In many animals no foreign deposits were found in an entire section. In some embolic lesions small broken-off hair shafts, cut transversely, obliquely or longitudinally, were unmistakably visible in the stained sections and when examined under crossed Nicol prisms (Figs. 3, 4 and 5). In some

other lesions, the foreign material was not identified beyond surmise (Fig. 6).

It was concluded that all lesions in the lungs of the mice had been initiated by the adventitious introduction into the tail vein of foreign particles, which traveled thence to the right heart and were filtered out in the lungs. The final site of lodgment was obviously determined by the size of the particulate matter in the vascular system. Very minute fragments might reach the alveolar capillary bed and produce granulomas. The same lesions were found in the lungs of mice injected repeatedly with normal saline. The more often the mice were injected, the more numerous the lesions. The total number of lesions in the whole of the lungs must have been considerable in some mice.

Most of the lesions must have been embolic in origin, for there were undisputed changes on the arteriolar side of the pulmonary circulation. The most frequent ones were characterized by vasculitis, perivascular cuffing, fibrinous and cellular thrombi. Many of these contained parts of hairs, scales or unidentifiable material. A fragment of hair was attached to the wall of the right ventricle in one mouse, and in the right atrium of another. None of the embolic lesions were of such severity as to have caused infarction.

Comments

The lesions occurring in the lungs of mice must frequently follow the simple act of repeated intravenous injection of any solution. The larger the number of injections, the greater the number of lesions.

Since so many of the foreign-body lesions were found in tissues of the pulmonary circulation, it is unnecessary to consider other routes, e.g., aspiration. The latter is a possible event.[1] A consideration of the mouse tail, and standard practices of intravenous injections provide some explanation. Under the dissection microscope the epidermal scales on the mouse tail overlap like rectangular slates on a roof, and the hairs are fine bristles which are easily broken up (Fig. 7). Any skilled operator can perform as many as 50 to 60 intravenous injections in an hour, and many workers use needles which are neither new, sterilized, nor washed during injection of hundreds of mice. The repeated use of the same needle (27-gauge) soon results in accumulation of epidermal debris and broken-off hairs on its surface. Hence, it is not surprising to find the sequence of pathologic events encountered in this study.

By gently scraping mice tails and suspending the material in saline it was proved that some of it could traverse the lumen of a 27-gauge

needle, and in a small number of mice, lesions with foreign matter were produced. One rabbit was injected with hair and epidermal debris in suspension and sacrificed at the end of 5 days; granulomas and other lesions were found, but the injection, not unexpectedly, also caused areas of hemorrhagic infarction.

Summary

Fragments of hair, epidermal scales, and other unidentifiable extraneous matter caused embolic and granulomatous lesions in the lungs of mice, as a result of being accidentally introduced by injection into the tail vein. Repeated daily injections increased the incidence of these lesions. With standard practice of intravenous injection into mice, unsterilized or even unwashed needles are commonly used, and often for a very large number of animals.

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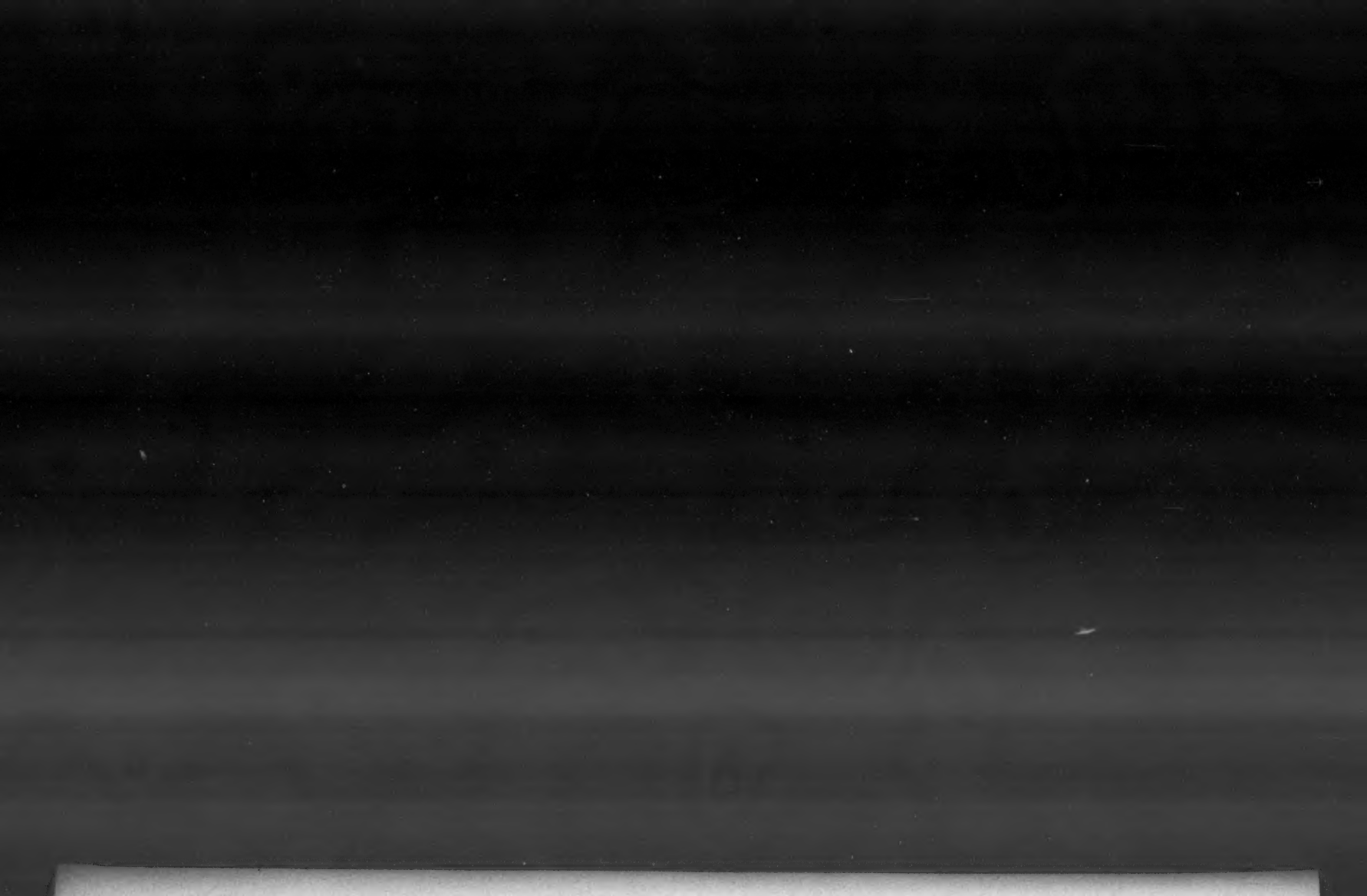
LEGENDS FOR FIGURES

FIG. 1. Mouse injected daily 10 times. Lung shows granulomatous lesion and an arterial embolus but no obvious foreign body. Hematoxylin and eosin stain. × 110.

FIG. 2. Mouse injected 5 times. Lung shows a granuloma with a crevice in the center. Hematoxylin and eosin stain. × 230.

FIGS. 3 and 4. Lungs of 2 separate mice. Each received injections once a day for 10 to 15 days. Hair and emboli in arterioles with some perivascular reaction. Hematoxylin and eosin stain. × 230.



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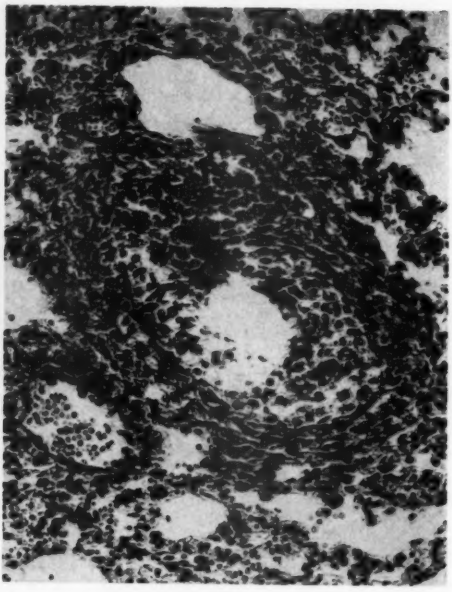

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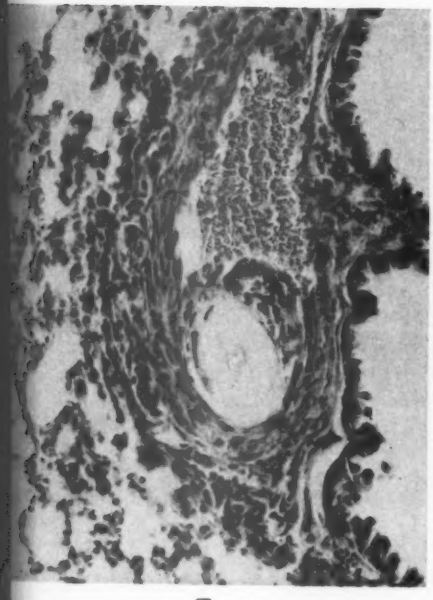

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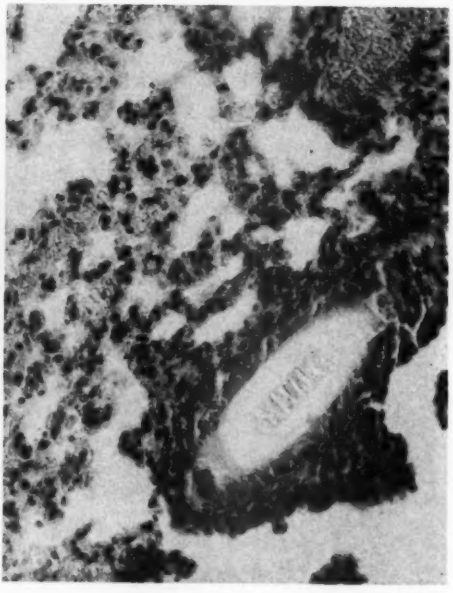

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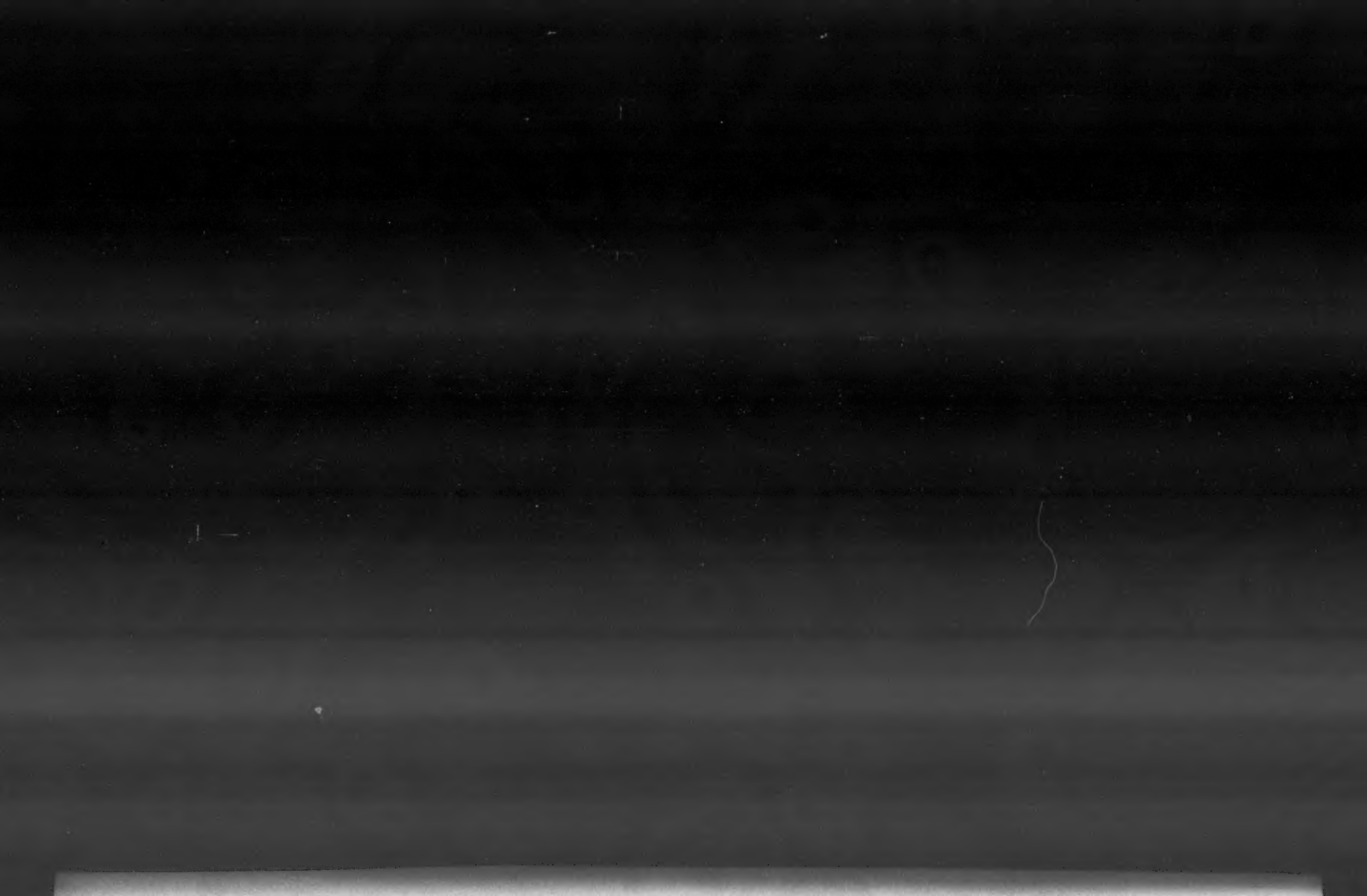
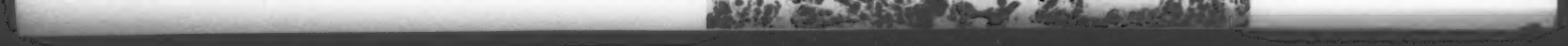
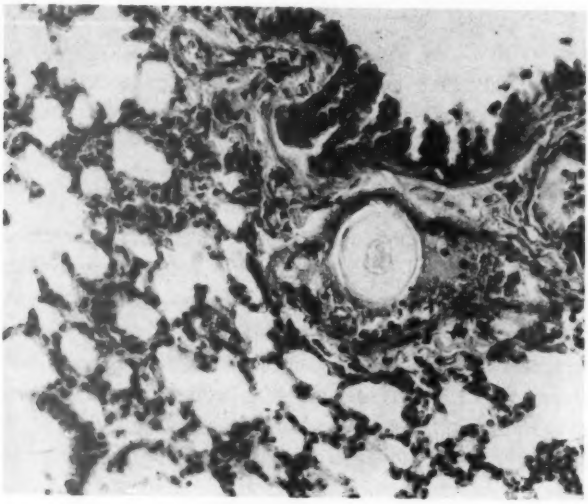
FIG. 5. Mouse lung. Animal received daily injection for 10 days. Fragment of hair in small blood vessel. Hematoxylin and eosin stain. × 230.

FIG. 6. Mouse injected 10 times with normal saline. Lung shows granuloma with fiber of unidentified nature in the center. Hematoxylin and eosin stain. × 230.

FIG. 7. Tail surface of 3 mice, showing arrangement of epidermal scales and fine bristles protruding from the seams. × 20.







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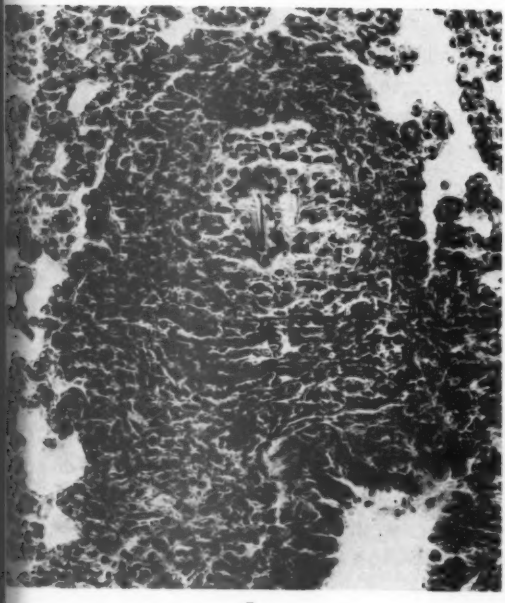

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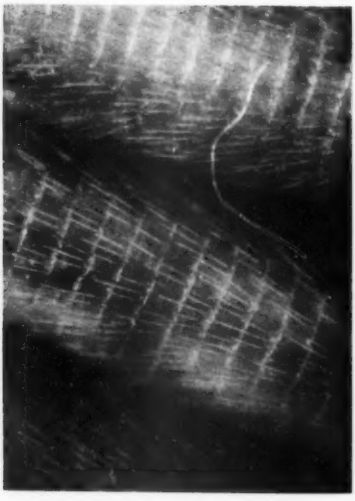

7

THE ASSOCIATION OF VASCULAR ANOMALIES WITH ANENCEPHALY

A Postmortem Study of Nine Cases in One of Which Unilateral Anencephaly Was Present in a Conjoined Double Monster *

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It has been clearly shown that artificial occlusion of a major cerebral artery in developing chick embryos regularly induces a marked retardation in the differentiation of the neuroectoderm and in the development of the brain.[1] There is also abundant evidence that certain congenital malformations of the brain in human beings, notably anencephaly, are often associated with extensive anomalies of the major cerebral blood vessels.[1] It remains uncertain, however, whether vascular anomalies are generally responsible for malformation of the neuroectoderm, whether they result from the latter, whether some third etiologic factor is responsible for both, or indeed, whether the two malformations arise independently. The frequency of vascular anomalies in the cases of anencephaly previously studied in this laboratory[1] makes it seem likely, however, that the two anomalies are related in their development. To learn more about this possible relationship, the anatomic characters and the relative severity of each of these lesions has been precisely defined in 9 cases of anencephaly. One of the cases is unique, and it has special implications for the relationship between vascular anomalies and anencephaly, for in it, a well formed cranium and brain and an anencephalic head with vascular anomalies were found together in a conjoined double monster of *duplicitas anterior* type, the product of enzygotic twinning.[2]

Summary of Necropsy Observations in a Case of a Conjoined Double Monster with Unilateral Anencephaly

The conjoined twin weighed 2,900 gm. and measured 42 cm. when delivered dead, at term, after an uneventful pregnancy, to a healthy 30-year-old Para I, gravida III. The maternal serological test for syphilis was negative; the Rh factor was present. No family history of twinning could be elicited.

The female child had two heads, situated side by side, each properly

* This investigation was supported by a research grant from the National Institute of Neurological Disease and Blindness of the National Institutes of Health, Public Health Service.
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aligned with the body axis and attached by a short neck to a single torso. The four extremities were normally developed. The left head was of approximately normal size and was well formed (Figs. 1 and 2). The calvarium and cranial cavity were normal. The brain weighed 303 gm. and showed no notable gross or microscopic structural anomalies (Fig. 3). Several small recent subarachnoid hemorrhages were present over the occipital lobes. Two normal-sized internal carotid arteries and two vertebral arteries penetrated the base of the skull and united through a long basilar artery to form a well defined circle of Willis. The cerebellum and brain stem were well developed, as was the spinal cord. There was a rachischisis with overlying skin defect in the thoracolumbar region.

The face and the inferior portions of the skull of the right head, although well-formed, were slightly smaller than those of the left. Two eyes were present with minimal proptosis. The calvarium was absent. The cerebrum was represented by a discoid mass of friable, extremely hemorrhagic tissue, in no way reminiscent of the normal brain; it weighed 69 gm. and measured 6 by 6 cm. across and 2 cm. in thickness. Many large and small tortuous blood vessels were grossly visible in and over this mass. There was no cerebellum. The brain stem and upper cervical portion of the spinal cord were incompletely formed, and were comprised principally of strands of vascular and fibroglial tissue. The lower portion of the spinal cord was of approximately normal size and was contained within an open bony canal that lay parallel and approximately 1 cm. lateral to that of the left twin. There was total rachischisis, with absence of the skin. The skin defects over the left and right spines merged in the thoracolumbar region (Fig. 2).

The cerebral mass was formed of islands of poorly differentiated and structurally underdeveloped neural tissue. There were a few small cysts lined in places by ependyma. Some contained choroid. The abundant fibroglial stroma was formed of excessive numbers of fibroblasts and a few astrocytes, oligodendroglia, and microglia-like cells. There were rich plexuses of abnormal, large and small, thin-walled veins, and also many individual thick-walled arteries, both in continuity with abnormal vessels over the medulla and upper portion of the spinal cord. No cerebellar tissue could be identified. The thoracolumbar portion of the spinal cord was normally formed and contained well differentiated gray and white matter. Many dorsal root ganglia were present. Myelin was sparse in the spinal cord but was present in approximately normal amounts in the dorsal roots.

The single large thorax contained two pairs of lungs each with a well

developed trachea. A large, 40 gm. globular heart occupied the mid-left portion of the thorax; it had 8 chambers and multiple septal defects. There were two aortas. One, with a circumference of 2.2 cm., arose from a principal ventricle; it arched in normal fashion in the left upper thorax and descended into the abdomen. Innominate, common carotid and subclavian arteries, with circumferences respectively of 10, 6, and 3 mm. took origin from the arch of this aorta and provided the left head with two large carotid and two vertebral arteries. A second aorta, with a circumference of 2.8 cm., stemmed from a rudimentary ventricle and passed across the right upper quadrant of the chest. A single branch, measuring 3 mm. around, entered the base of the right skull; it could not be identified in the cranial area. The terminal portion of this aorta coursed toward the left and divided. The smaller branch entered the soft tissues of the left neck; the larger penetrated the base of the left skull. The abdominal viscera showed duplication of the stomach, excessive quantities of liver (80 gm.) with supernumerary lobes, and a bicornate uterus.

Summary of Necropsy Observations in Eight Cases of Anencephaly

To learn more about the possible relationship between the angiomatous and neural malformations in anencephaly, the anatomic distribution of each of these lesions was studied by means of detailed histologic examinations in eight additional cases. In each instance, serial, coronal sections were made through the cranial mass and through the medulla and spinal cord, at all levels. These tissues were embedded in celloidin and sectioned at 10 to 30 μ for staining by hematoxylin and eosin, Masson's trichrome method, Nissl's cresyl violet method, and Weil-Loyez's method for myelin. Selected portions were embedded in paraffin and stained by Verhoeff's method for elastic tissue. In addition, frozen sections were stained by Hortega's silver carbonate method for astrocytes, oligodendroglia, and microglia.

The cerebral deformities were grossly characteristic in all eight cases.[8] Proptosis of both eyes was regularly present. Each had well formed and well preserved retinas and often rudimentary segments of optic nerve. The calvaria were absent (Fig. 4). The cerebral hemispheres were regularly replaced by hemorrhagic, friable masses which, as shown by histologic examination, were composed of poorly differentiated neural tissue with large and small cysts lined partially by ependyma. All were haphazardly arranged in dense fibroglial tissue. Numerous engorged, thin-walled blood vessels were regularly present

TABLE I

Summary of Necropsy Observations in Nine Cases of Anencephaly, One a Conjoined Twin

Case No.	Birth weight gm.	Duration	Sex	Neural malformation	Vascular malformation	Other conditions
1	2900	Born dead	F	Duplication of central nervous system. Normal left brain, 303 gm. Right cerebrum replaced by 69 gm. mass of hemorrhagic tissue 6 x 6 x 2 cm. (Figs. 1–3).	Right brain supplied by large vertebral and carotid vessels. Left cephalic area supplied by small arterial branch from rudimentary, duplicated aorta. Plexus of abnormal blood vessels in cerebral mass and about cervical cord.	Conjoined double monster of *duplicatas anterior* type. Single thorax with 8-chambered heart. Duplication of lungs, esophagus, and stomach. Excessively lobed liver. Bicornate uterus.
2	3600	6 hours	M	Calvarium absent. Cranial mass of hemorrhagic tissue weighing 78.4 gm. Formed of undifferentiated neural, glial, and mesenchymal tissue. Medulla and cord well formed (Fig. 4).	Dense plexus of dilated small and large vessels in cerebral mass extending in lesser concentrations over medulla and throughout spinal canal. Vessels without muscular or elastic tissue. (Figs. 5–7).	Hypoplasia of adrenals (1.1 gm.). Hyperplasia of thymus (21.9 gm.).
3	3450	Born dead	M	Cranial vault absent. Hemorrhagic mass 6.5 cm. across and 2 cm. thick, attached to well formed medulla.	Plexus of abnormal blood vessels in cerebral mass, about medulla, and extending throughout spinal canal.	Hypoplasia of adrenals (0.8 gm.). Hypoplasia of right kidney. Hyperplasia of thymus (20.5 gm.).
4	2460	4 hours	M	Calvarium absent. Discoid cerebral mass, 5 mm. thick, over entire base of skull. Medulla and cord normal.	Numerous sinusoidal vessels in cerebral mass. Markedly abnormal vessels in spinal canal.	Hypoplasia of adrenals (0.4 gm.). Hyperplasia of thymus (18 gm.).
5	2460	4 hours	M	Calvarium absent. Two 2-cm. hemorrhagic masses on base of skull attached to medulla.	Dense plexus of vessels in cephalic mass extending over medulla and cord.	Hypoplasia of adrenals (1.5 gm.). Hyperplasia of thymus (18 gm.).
6	2480	2 hours	F	Cranial vault absent. Discoid cerebral mass 5 mm. in thickness. Medulla and cord present.	Many abnormal blood channels throughout cerebrum. Vessels not notably abnormal in spinal canal.	Hypoplasia of adrenals (0.5 gm.). Atresia of urethra.
7	2460	8 days	M	Calvarium absent. Cerebral mass weighing 26.8 gm., measuring 6 x 6 x 1.5 cm., attached to small medulla without cerebellum or pons.	Numerous thin-walled blood vessels throughout cranial mass. Normal vessels in spinal canal.	Hypoplasia of adrenal (0.4 gm.). Stenosis of extra-hepatic bile ducts. Cleft palate.
8	1570	1 hour	M	Calvarium absent. Hemorrhagic friable mass 2 x 1 x 1 cm. in cranial area. Medulla and cord present.	Many abnormal blood vessels in cerebral mass extending on to medulla but not into spinal canal.	Hypoplasia of adrenal (0.4 gm.).
9	1420	Born dead	F	Cranial vault not formed. Cerebral mass 2 x 2 x 1 cm. Medulla and cord present. Cerebellum and pons absent.	Dense plexus of thin-walled vessels in cerebral mass. No extension of vascular anomaly into spinal canal.	Hypoplasia of adrenal (1 gm.).

throughout the cranial mass (Fig. 5). The vascular channels were formed of an endothelial lining with very slender fibrous walls. These were devoid of muscular and elastic tissue. The adventitia was sparse (Fig. 6). The medulla and cervical regions of the spinal cord were partially or totally developed and were regularly attached to the cranial mass. Cerebellar tissue was rarely present and hemispheres were never formed.

In four cases the angiomatous plexus, grossly conspicuous in the cerebral mass, extended in lessening degree over the medulla and about the entire spinal cord (Fig. 5). This tissue was formed of variably sized, thin-walled, vessels arranged as a cirsoid mass, occupying principally the spinal leptomeninges (Fig. 7). Some racemose vessels also extended into the medullary substance of the cord. The vessels, regularly lined by endothelium, had very slender fibrous walls that contained neither muscular nor elastic tissue. The channels lay side by side with little intervening adventitia or stroma. The spinal angiomatous deformity was clearly in continuity with, and bore a conspicuous structural similarity to, that in the cranial area. In general, the vascular channels were smaller and less numerous in the spinal canal. The neural tissues of the thoracolumbar portion of the cord, and often those of the cervical and medullary portions, as well as the dorsal roots and dorsal root ganglia, were well developed and well preserved. Many neurons, some astrocytes and oligodendroglia, and partially myelinated axons were present. Myelination was regularly most advanced in the posterior fasciculus and dorsal roots.

The necropsy observations in the nine cases of anencephaly are summarized in Table I.

Discussion

The occurrence of a characteristic case of anencephaly in one monovular twin provides conclusive evidence that, in this instance, the cerebral deformity did not arise from an intrinsic defect in the anlage of the neuroectoderm or in "defective organization of the primordial tissue," as has been postulated.[4] Furthermore, the presence in most instances of anencephaly of well formed and well preserved structures that originate from migratory neuroblastic tissue (the retina and the intra-osseous portions of cranial nerves and cranial nerve ganglia[1,2,5]) makes it plain that the initial formation of the encephalon is generally normal, through at least the first 6 or 7 weeks of fetal life. It is noteworthy that this period, especially from the 5 mm. to the 22 mm. stage, is not only characterized by rapid development and differentia-

tion of the neuroectoderm but also by equally active angiogenesis. The primordial vascular bed becomes organized, with the establishment of anastomoses between many isolated channels, with absorption of many others, and finally, with completion of the circle of Willis and integration of the cerebral circulation into the systemic vascular system.[6]

The regular association of the vascular and neural deformities in anencephaly is well established. In consideration of the pathogenesis of these congenital malformations, the possibility presents itself that a single factor, or several independent factors simultaneously, may alter neural development and influence angiogenesis. The regularity in the association of these two anomalies, their very intimate anatomic relations, and the close parallel in their severity from place to place in the cephalic area, constitute strong evidence that they do not take origin from totally unrelated causes and develop independently. On the other hand, the frequent dissociation of the neural and angiomatous lesions throughout the spinal canal makes it unlikely that both anomalies arise from a common cause.

The possibility also remains that maldevelopment of the neuroectoderm could itself initiate abnormal angiogenesis. However, again, the occurrence of vascular anomalies in the spinal canal, in the presence of normal neural development, provides conclusive evidence that such is not the sequence of events in this area. Furthermore, the very close continuity and the conspicuous morphologic similarity of the vascular anomalies in the spinal and cephalic areas provides strong inferential evidence that the same situation also obtains in the cranial region.

The conspicuous neural deformities that have been induced in chick embryos by occlusion of one or another of the cerebral blood vessels, are manifestations of the critical dependence that the neuroectoderm has, for its differentiation and development, upon an adequate blood supply. The characteristics of anencephaly in human beings, when considered in light of these experimental observations, give strong support to the likelihood that abnormal angiogenesis is directly responsible for the development of anencephaly.

Summary

The morphologic features of a conjoined double twin with one normally developed cranium and brain and one anencephalic head clearly show that in anencephaly the anlage of the neuroectoderm has the potentiality for normal development and that, in fact, the initial development of the encephalon is normal during the early few weeks of fetal life.

Detailed studies of eight additional cases of anencephaly disclosed that in each instance there were conspicuous neural and angiomatous anomalies in very close anatomic association throughout the cranial mass. In four cases the angiomatous lesions extended in continuity, but in diminishing severity, about the entirety of the well-formed spinal cords.

The findings are in agreement with previous experimental observations and provide additional evidence that abnormal angiogenesis plays an important causative role in the pathogenesis of anencephaly in human beings.

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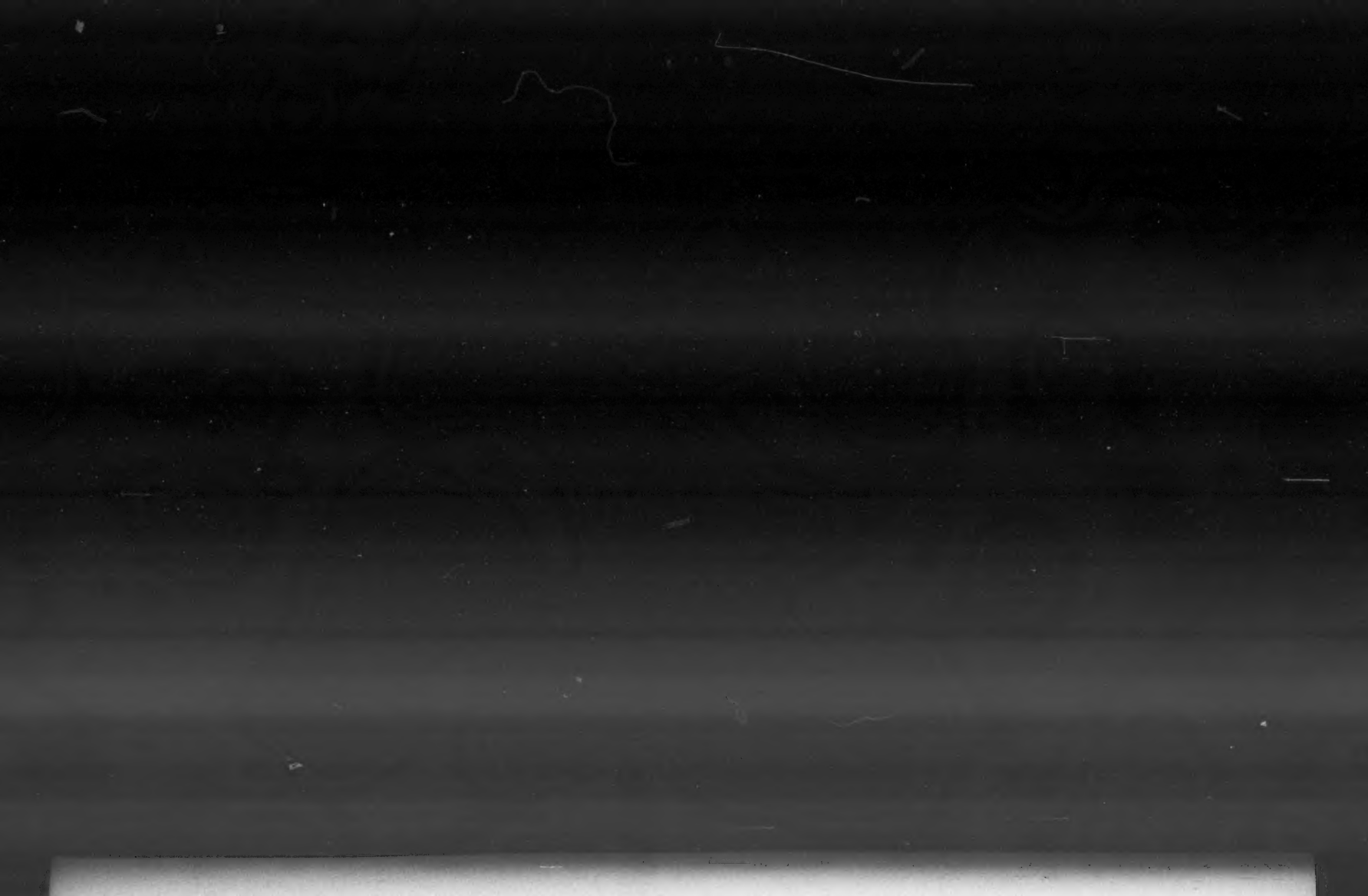
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Case 1. A conjoined twin with one normally developed head and brain and one anencephalic head.

FIG. 2. Case 1. Posterior aspect. The cerebrum of the right head is represented by a sessile mass of soft hemorrhagic tissue. The medulla and upper cervical portion of the cord are poorly developed. A fibrous tissue membrane covering has been removed from the well-formed thoracolumbar segment of the spinal cord. The normally-developed left spinal cord occupies a parallel bony canal. The skin defect merges in the thoracolumbar region and extends into the right cervical area.



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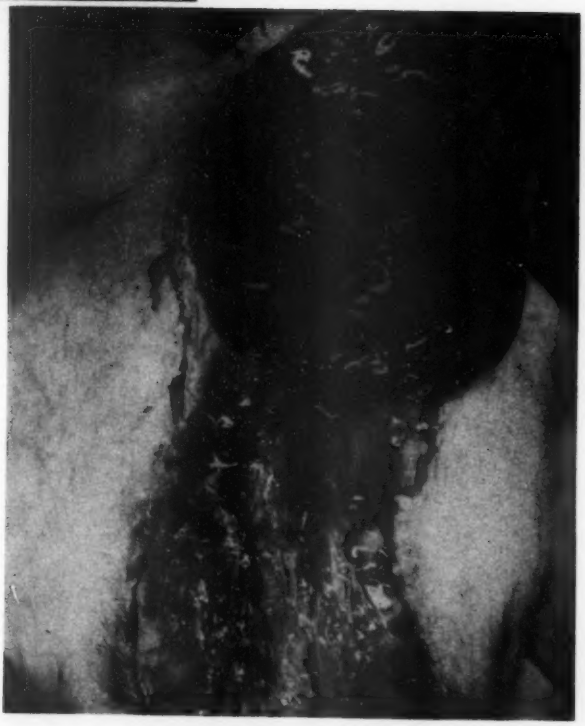

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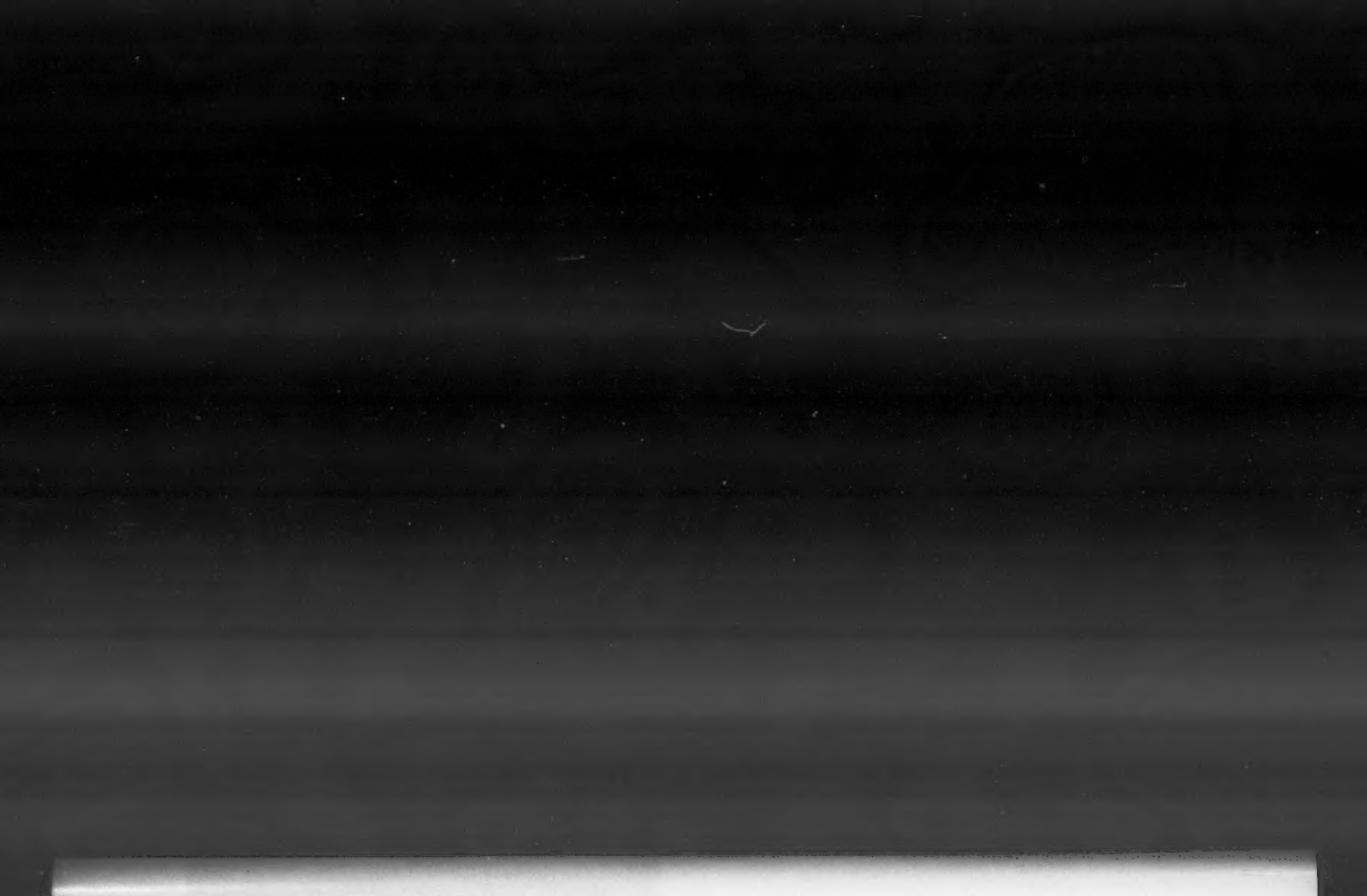
FIG. 3. Case 1. The brain and spinal cord of the left head are normally developed. The right cranial mass, in no way reminiscent of a brain, consists of undifferentiated neural, glial, and fibrous tissue. The thoracolumbar segment of the cord is small but well formed. Many abnormal blood vessels are visible in and over the cranial mass. These extend into the cervical region of the cord.



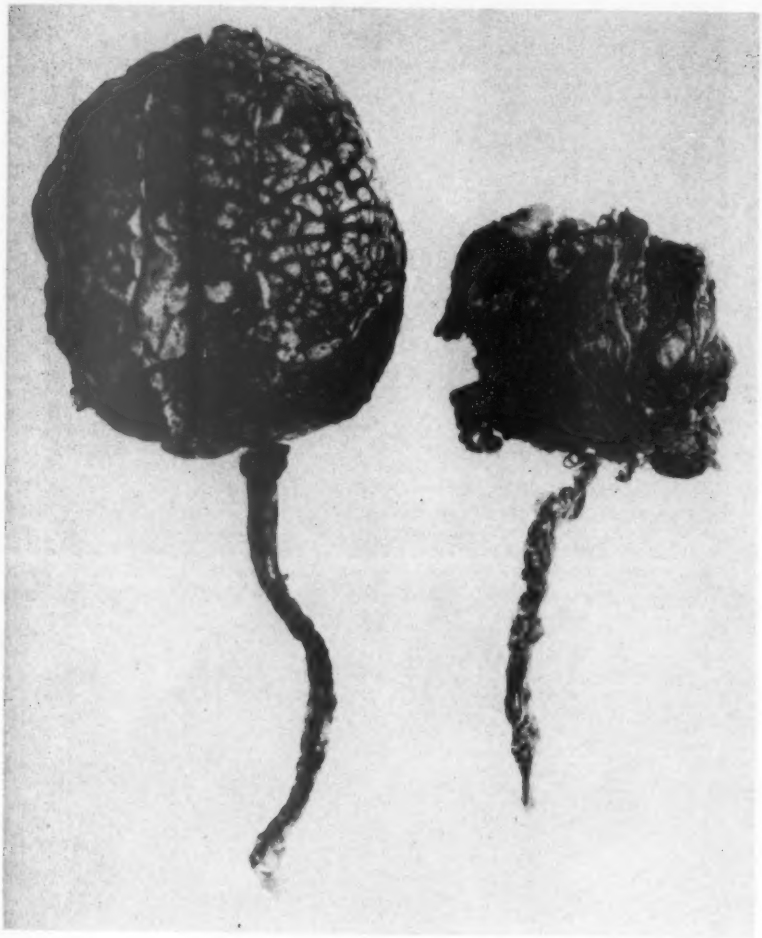


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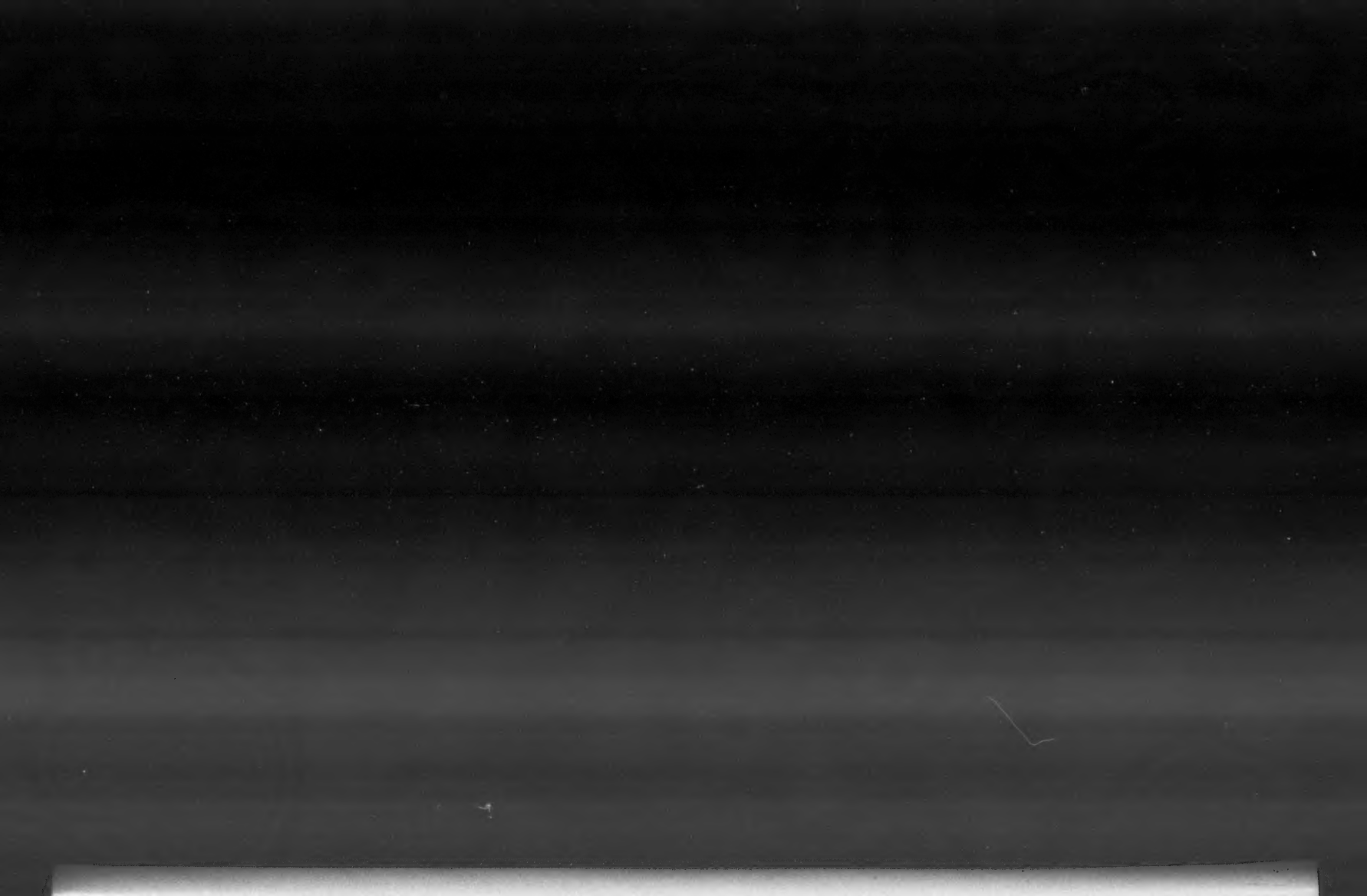
FIG. 4. Case 2. Anencephaly characterized by absence of the calvarium and replacement of the cerebrum by a mass of hemorrhagic tissue.

FIG. 5. Case 2. The cranial mass is attached to a well formed medulla and spinal cord. Dense plexuses of abnormal blood vessels in the cranial mass are in continuity with a racemose layer of abnormal vessels over the medulla and cord.



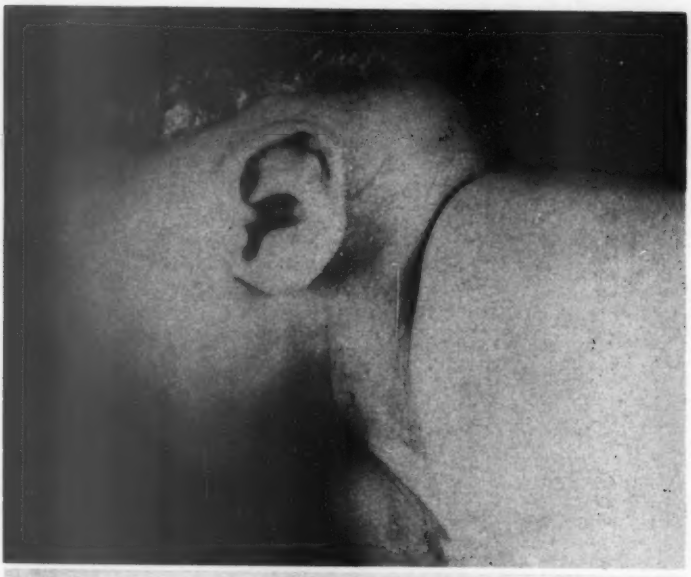


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FIG. 6. Case 2. A dense maze of abnormal blood vessels, characteristic of those present throughout the cerebral mass. The vessels are lined by endothelium. Their thin fibrous walls contain neither muscular nor elastic tissue. Islands of undifferentiated neural tissue border the angiomatous mass. Hematoxylin and eosin stain. × 100.

FIG. 7. Case 2. The leptomeninges in the lumbar region of the spinal cord contain a dense plexus of abnormal blood vessels. A lesser number are also present in the well formed spinal cord. The angiomatous anomaly is morphologically identical to that in the cerebrum, although notably less severe. Hematoxylin and eosin stain. × 20.

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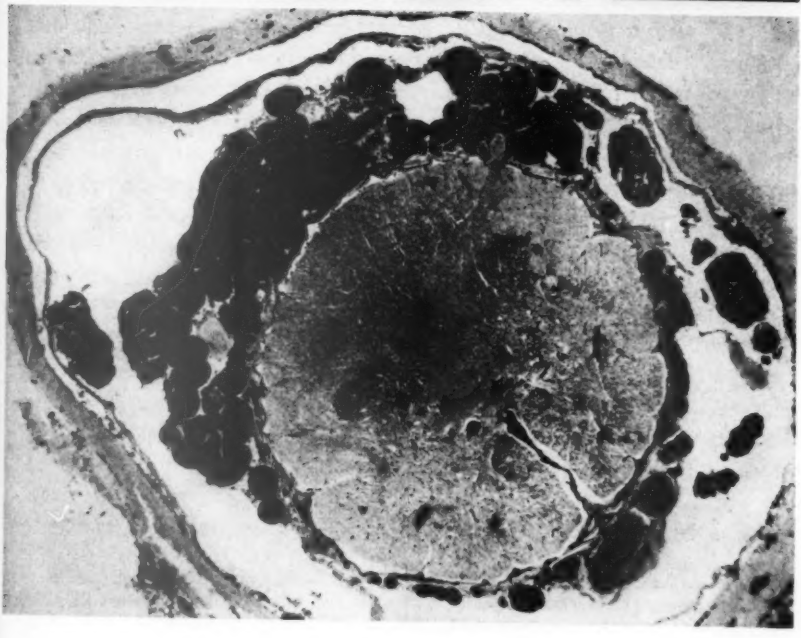

7

THE NEUROPATHOLOGY OF HEREDITARY OPTIC ATROPHY (LEBER'S DISEASE); THE FIRST COMPLETE ANATOMIC STUDY*

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For more than 85 years since Leber's paper in 1871,[1] several thousand cases of the disease termed hereditary optic atrophy (Leber's disease) have been reported in the medical literature, but not a single complete examination of the central nervous system in such a case has been described. In 1930, Rehsteiner,[2] utilizing modern histologic techniques, examined the retina and optic nerves procured from one patient at necropsy. He noted destructive changes in the ganglion cell layer of the retina and irregular demyelination of the optic nerves without round cell infiltration. The demyelination was most striking in the papillomacular bundle, but was not confined to this region.

Several postulations regarding the pathogenesis of this disease have been advanced. Brief mention only is made of the hypotheses of pituitary gland disorder,[3,4] chronic arachnoiditis about the optic nerves,[5,6] toxic retrobulbar neuritis due to neighboring infection, and primary neuronal degeneration localized in the retina and optic nerve.

Much has been written concerning certain neurologic disorders which occur concomitantly with Leber's disease. Leber himself noted that minor neurologic manifestations, such as headaches, vertigo, and epileptiform attacks occurred among members of the families of some of his patients. He described these people as constituting a neuropathic type, and thought that they were prone to develop disease. Some of the more commonly described associated disorders were epilepsy,[7,8,9] hysteria, mental impairment, nystagmus, hand tremors,[10] absent knee or ankle reflexes, aching pains, slight loss of sphincter control, signs of pyramidal tract disease,[11] ataxia, and sensory disturbance.[12] In addition, it should be remarked that optic atrophy has frequently been reported in patients with Marie's and Friedreich's ataxia. It should also be noted that one patient with Leber's disease had clubfoot similar to that seen in Friedreich's ataxia. This apparently had been inherited from the mother.[13]

It is not the purpose of this paper to discuss the clinical aspects of

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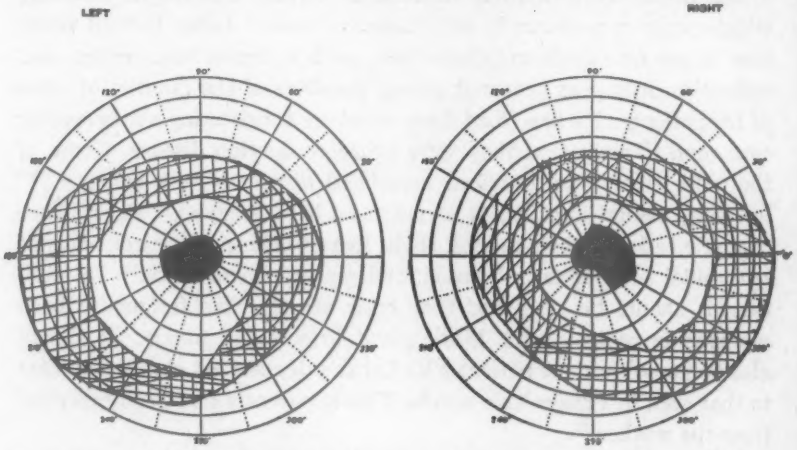
Leber's disease. There are many excellent published contributions in this field.[14-20] It is the intention to present for the first time the results of a complete anatomic study of the central nervous system in a patient with this disorder.

Report of a Case

A 58-year-old white male newsstand operator first noted bilateral loss of vision at the age of 17 years. This progressed rapidly to a maximum point over a period of 2 to 3 weeks and remained stationary thereafter. There was no history of headache, vertigo, epileptiform attacks, mental change, alcoholism, or excessive smoking.

A 60-year-old brother, and a maternal uncle had had similar illnesses. The uncle died at 65 years of age from a heart ailment, and had no other neurologic difficulties. The brother, also a newsstand operator, was not hospitalized, but the authors had the opportunity to examine him thoroughly on several occasions. The onset of his eye disease was at 18 years, with a clinical history similar to that of the patient. The neurologic examination disclosed impairment of position sense in the toes, and there was also optic atrophy. The vision in each eye was 4/300. Visual fields (Text-fig. 1) showed changes consistent with those observed in Leber's disease. A fine horizontal nystagmus was



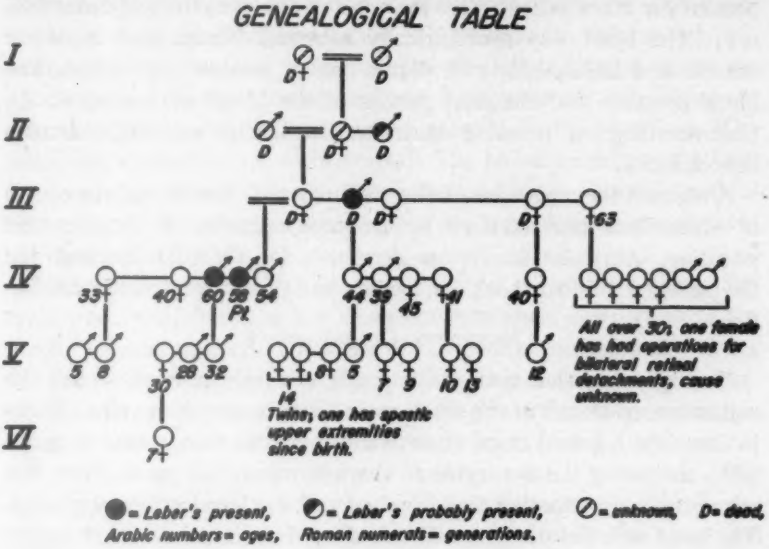
Text-figure 1. The visual fields of the patient's brother are shown. The cross-hatched area reveals the loss of peripheral field for a 5/330 isopter. There is a dense central scotoma to the 10 mm. test object.

present on lateral gaze. He was well nourished and well developed and had normal feet. There was no evidence of heart disease.

As shown in the genealogical chart (Text-fig. 2), the diagnosis of hereditary optic atrophy was made in all 3 members of the family, the

patient, his brother, and his maternal uncle. The members of the side of the family not depicted had no evidence of this condition.

Since birth, the patient had had a right clubfoot, a congenital dislocation of the right hip, and an undescended right testis. He had managed to walk well with the aid of special shoes until he sustained a fracture of the left mid-femur following an automobile accident at the



Text-figure 2.

age of 48 years. After being on crutches for some time, he finally managed to walk with the help of a cane. For several years he suffered from heart failure and had several admissions to hospitals because of the cardiac condition. There was no history of hypertension or renal disease. He was admitted finally to Montefiore Hospital because of rapid progression of heart failure.

Neurologic examination disclosed that the patient could see light and movement with his left eye, and could count figures at a distance of one foot with his right eye. Funduscopic examination revealed small, well outlined, round, pale gray-white discs characteristic of optic atrophy. There were relatively few disc vessels; well defined physiologic cupping was noted. No retinal abnormalities were seen. The pupils were equal, regular, and reacted simultaneously to light. A fine vertical nystagmus was noted on left lateral gaze. No visual field studies were performed. The other cranial nerves were normal. The remainder of the neurologic examination was normal except for bilateral

impairment of position sense in the toes. A right clubfoot was present (*talipes equinovarus*). The patient was emaciated and had a moderate degree of atrophy of the skeletal musculature.

All the symptoms and signs of severe congestive heart failure were present. Numerous large, dilated, vascular channels were present within the subcutaneous tissues of the trunk (these vessels had been present for many years before the onset of the symptoms of heart failure). The heart was concentrically enlarged. There were moderate anemia and leukopenia, with diminution of mature neutrophils. The blood pressure and chemical studies of the blood were normal. An electrocardiogram revealed auricular fibrillation and right bundle branch block.

A regimen for congestive cardiac failure was followed, and the course of illness was characterized by frequent episodes of dyspnea and vomiting. Auricular fibrillation persisted. On the 24th hospital day the patient exhibited shock for 4 hours and died in ventricular fibrillation.

Necropsy Observations

Necropsy revealed that the vascular channels present within the subcutaneous tissues of the trunk were dilated anomalous veins. Within the right inguinal canal there were numerous firm, rubbery, gray-white masses of tissue varying in size and measuring up to 5 cm. On microscopic examination these proved to be sclerosing hemangiomas. The heart weighed 620 gm. and was dilated concentrically and hypertrophied. No significant evidence of arteriosclerosis, scarring, valvular lesion, congenital anomaly, inflammation, or other abnormality was detected on careful gross and microscopic examination. The lungs, liver, spleen, and adrenal glands exhibited a striking degree of chronic passive congestion. The kidneys were essentially normal.

It was concluded that the patient had probably developed the enlarged heart and congestive heart failure as a result of the burden imposed on the heart by the large and abundant vascular channels and tumors present. However, no definite arteriovenous communications could be demonstrated.

Eyes. The eyes were normal in size and shape; the globes had the usual contour and measured 24 by 22 mm. Pathologic lesions were restricted to the retina and optic nerve. The former revealed peripheral cystic degeneration and the ganglion cell layer was strikingly atrophic. Occasional ganglion cells had pyknotic nuclei. A moderate degree of atrophy of the inner nuclear layer was also seen (Fig. 4).

The optic disc had a shallow excavation which was partially filled by

glial and connective tissue. Connective tissue was attached to the retina which had been displaced into the disc. The optic nerve was extensively atrophic, and there was marked thickening of the glial septa. No inflammatory exudate was seen (Fig. 2).

Brain. The venous sinuses and dura mater were normal. The cerebral hemispheres were symmetric and had well developed sulci and gyri. There was no evidence of increased intracranial pressure. The leptomeninges were thin and translucent. There were no thickenings, adhesions, or inflammatory lesions within the leptomeninges about the optic chiasma. The pituitary gland and fossa were normal. However, the optic nerve, chiasma, and tracts were diffusely shrunken. The remaining cranial nerves were normal. The blood vessels at the base of the brain disclosed no significant abnormalities. Coronal sections through the cerebral hemispheres, brain stem, and cerebellum revealed no macroscopic lesions.

Microscopic preparations of the optic nerves, optic chiasma, and optic tracts exhibited a severe degree of symmetric destruction of the myelin sheaths and axis cylinders in the central locations containing the fibers for macular vision. There was a relative degree of preservation of the peripheral fibers (Figs. 6-9). No inflammatory exudate was seen, but a minimal amount of free fat was present. Both geniculate bodies were shrunken and showed widespread and symmetric cellular atrophy. Neuronal cell loss and gliosis were present in all 6 layers (Figs. 11 and 12). Demyelination of the geniculocalcarine fibers was clearly demonstrated (Fig. 13). There were moderate amounts of free fat in these tracts. Careful examination of both calcarine cortices (Fig. 14) disclosed no significant cellular alterations. Microscopic study of the other cranial nerves, the cerebral hemispheres, cerebellum, and brain stem failed to show significant abnormalities. The pituitary gland weighed 750 mg. and was normal histologically.

Spinal Cord. A 39 cm. segment of the spinal cord with the attached cauda equina was available for examination. Multiple transverse sections at various levels failed to reveal abnormalities. Portions of the popliteal nerves and associated skeletal muscles appeared normal to the naked eye.

Microscopic study revealed a moderate degree of demyelination and gliosis in the gracilis columns, symmetrically situated within the cervical segment of the spinal cord (Fig. 15). Preparations of spinal cord at other levels failed to demonstrate further abnormalities. Myelin sheath destruction of slight degree was present in the peripheral nerves of the lower extremities (Fig. 17). There was no free fat present. The

skeletal muscles which were supplied by these nerves were the seat of a minimal degree of atrophy, characterized by shrinkage of the muscle fibers and proliferation of sarcolemmal nuclei (Fig. 16).

Discussion

The pathologic alterations in this case failed to lend support to earlier pathogenetic concepts of Leber's disease which presumed the presence of pituitary disorder, chronic arachnoiditis about the optic nerves, or toxic retrobulbar neuritis due to neighboring infection. Rather, there seemed to be a primary neuronal degeneration of the retina and optic nerve, with secondary degenerative changes in the remaining optic system except for the calcarine cortex. Transneuronal degeneration was clearly manifested within the geniculate bodies.

The peripheral neuropathy and the associated spinal cord changes were, in all probability, a direct consequence of the severe degree of malnutrition from which the patient suffered and were not at all related to his ocular disease. Although no relationship between the heredo-familial spino-cerebellar disorders and Leber's disease (hereditary optic atrophy) can be established from the data described above, the presence of both the position sense impairment in his brother and the right clubfoot in the patient leaves room for speculation.

Conclusion

For the first time a complete anatomic study of the central nervous system in a case of hereditary optic atrophy (Leber's disease) has been detailed. The data permit certain conclusions regarding the pathogenesis of this disease. These seem to point to a primary neuronal degeneration of the retina and optic nerve, with secondary degenerative changes of the remaining optic system except for the calcarine cortex. Attention is directed to certain parallel neurologic findings in cases of heredo-familial spino-cerebellar disorders.

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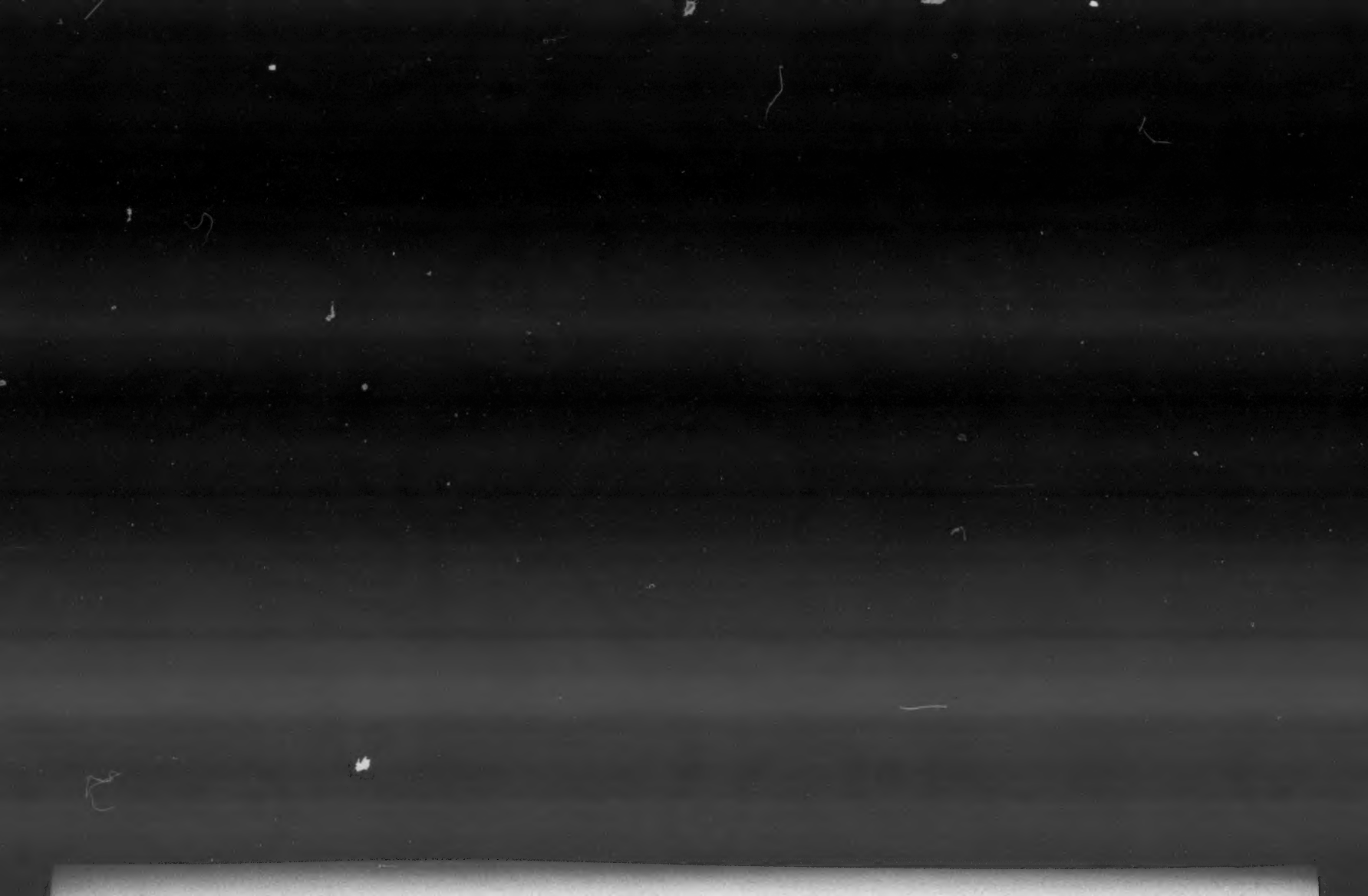
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. A normal optic disc. Hematoxylin and eosin stain. × 34.

FIG. 2. The patient's optic disc has a shallow excavation which is practically filled with glial tissue. The vessels show arteriosclerotic changes. The optic nerve is extremely atrophic, more evident on the temporal side. The glial septa are strikingly thickened. Weil stain. × 34.



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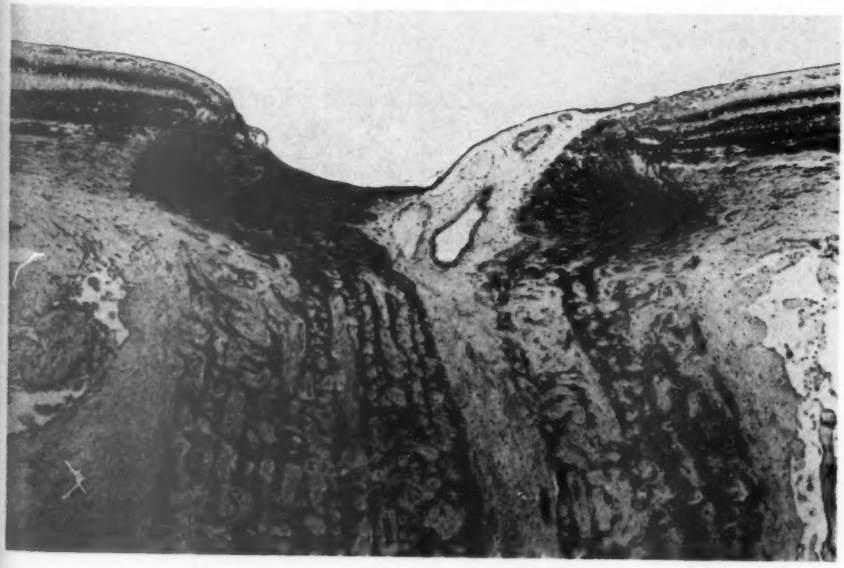

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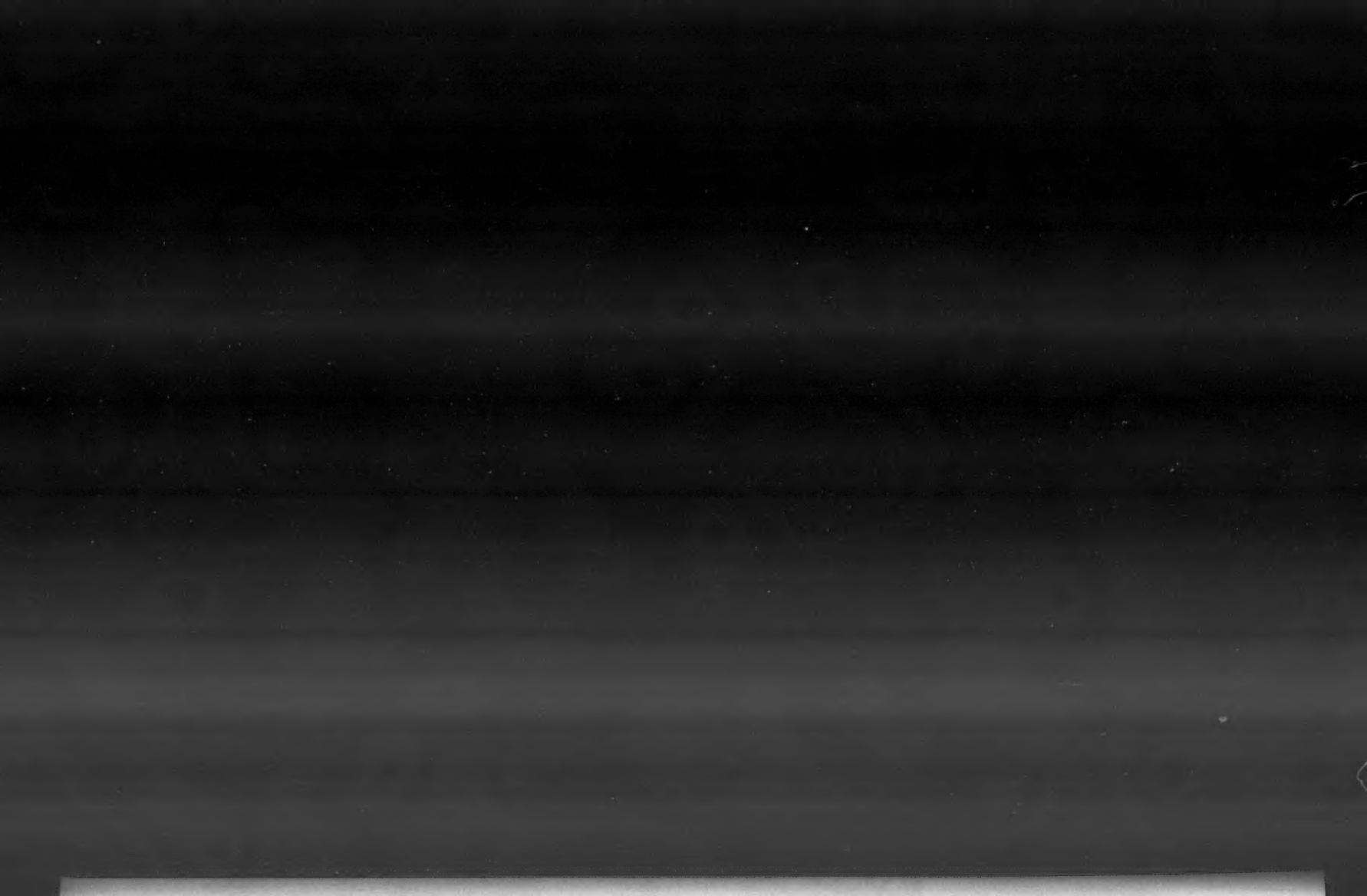
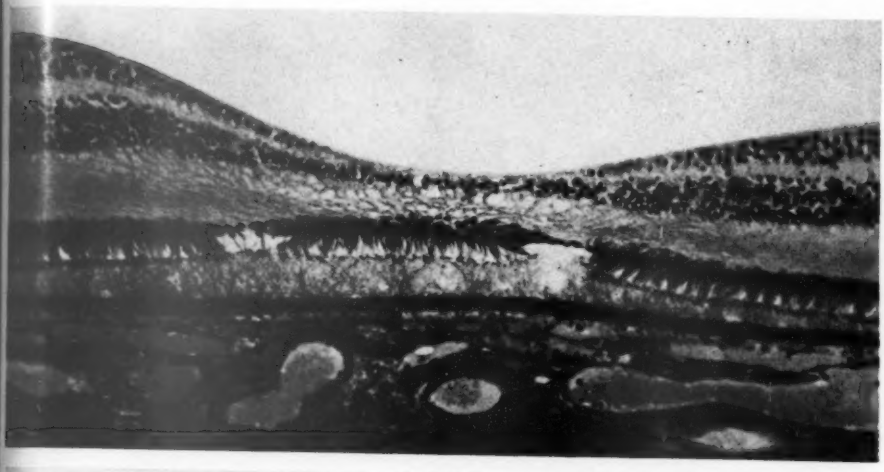
FIG. 3. A normal macula. Hematoxylin and eosin stain. × 128.

FIG. 4. The patient's macula exhibits a striking loss of ganglion cells, and a moderate degree of atrophy of the inner nuclear layer. Hematoxylin and eosin stain. × 128.





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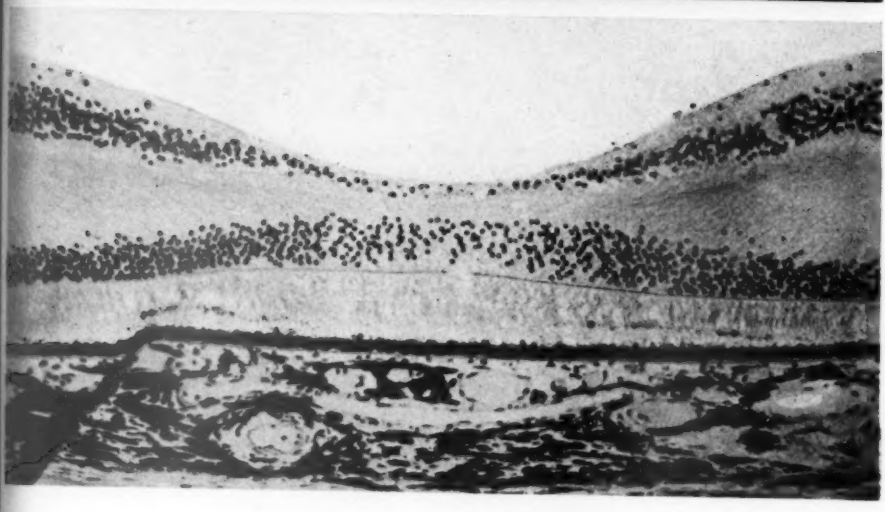

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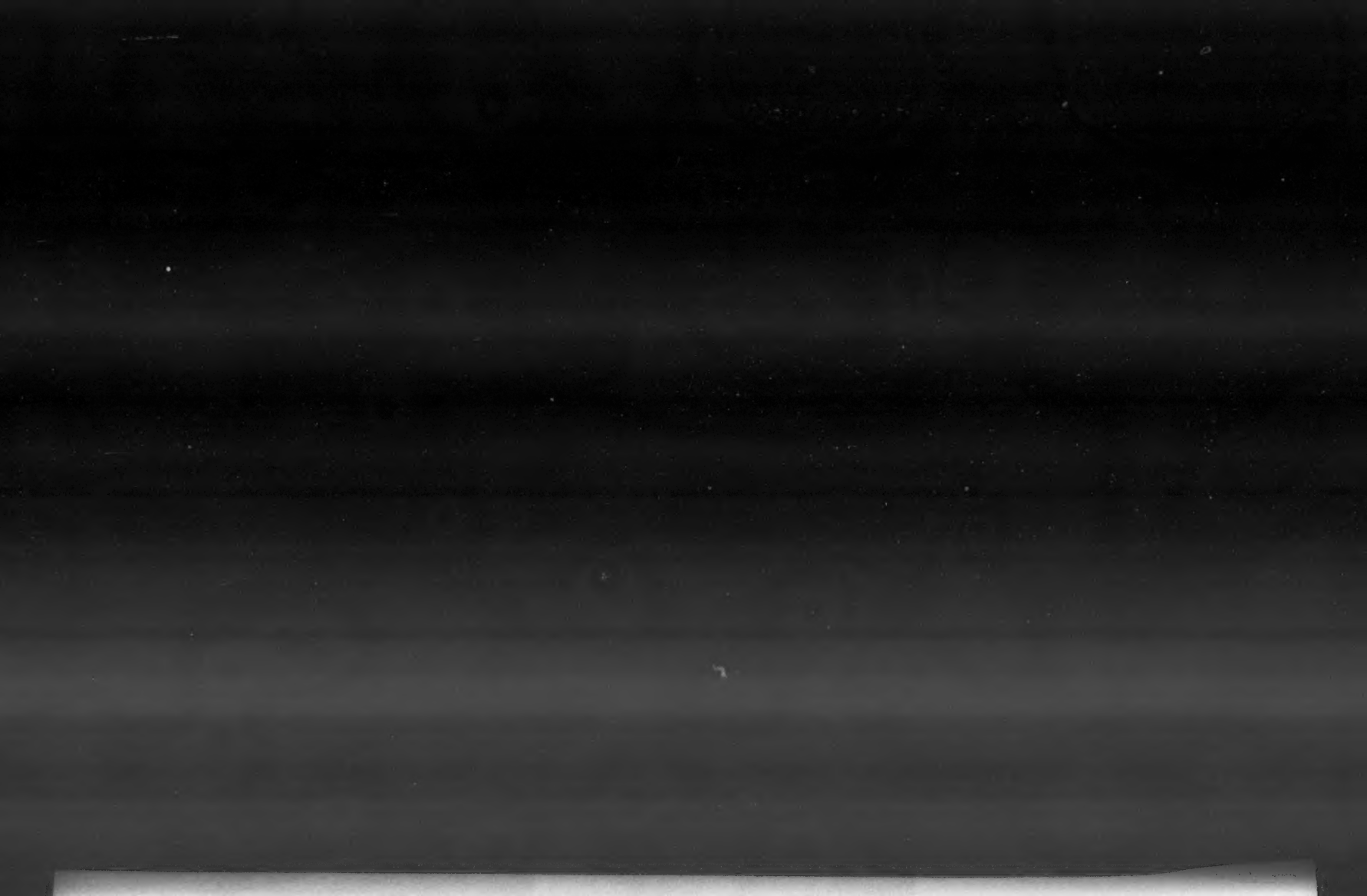
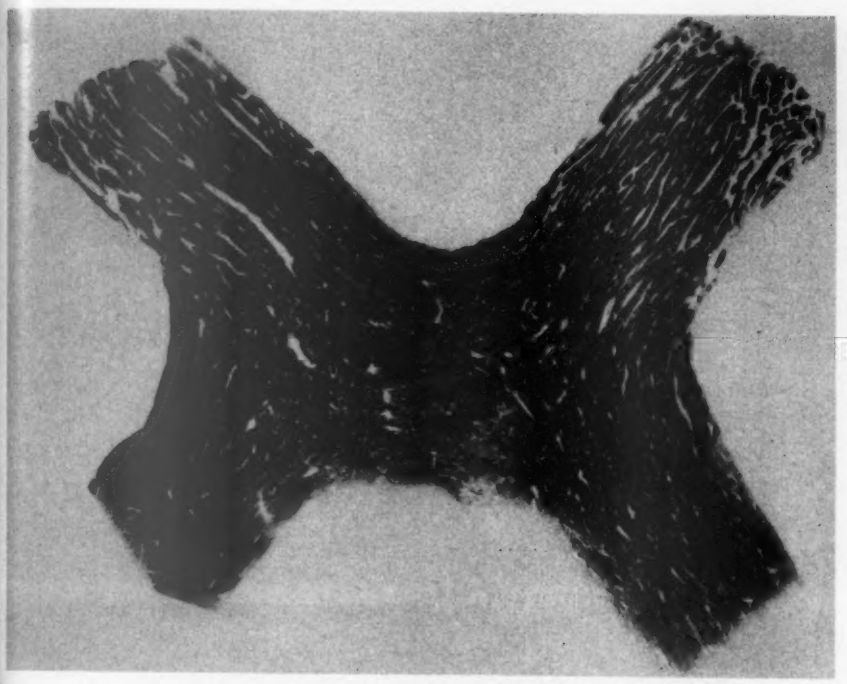
FIG. 5. Normal optic nerves, chiasma, and tracts are shown. Spielmeyer stain. × 8.

FIG. 6. The patient's optic nerves, chiasma, and tracts, showing severe diffuse demyelination. Spielmeyer stain. × 8.





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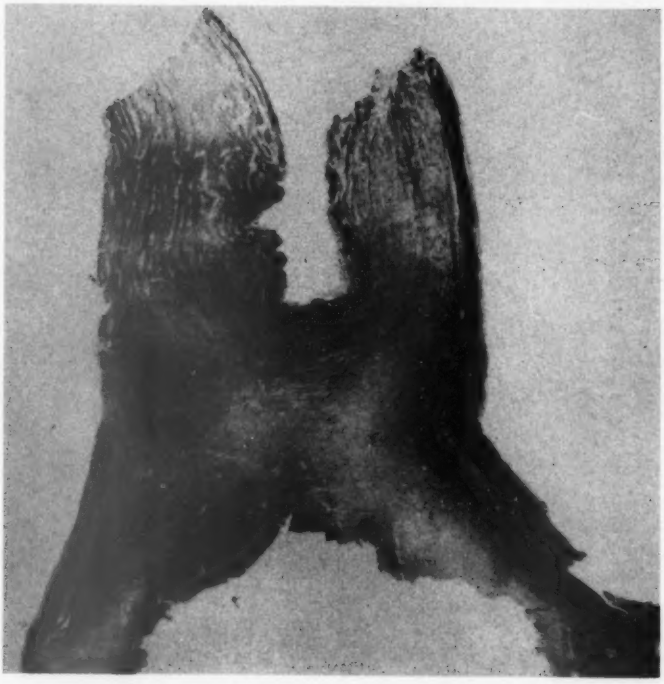

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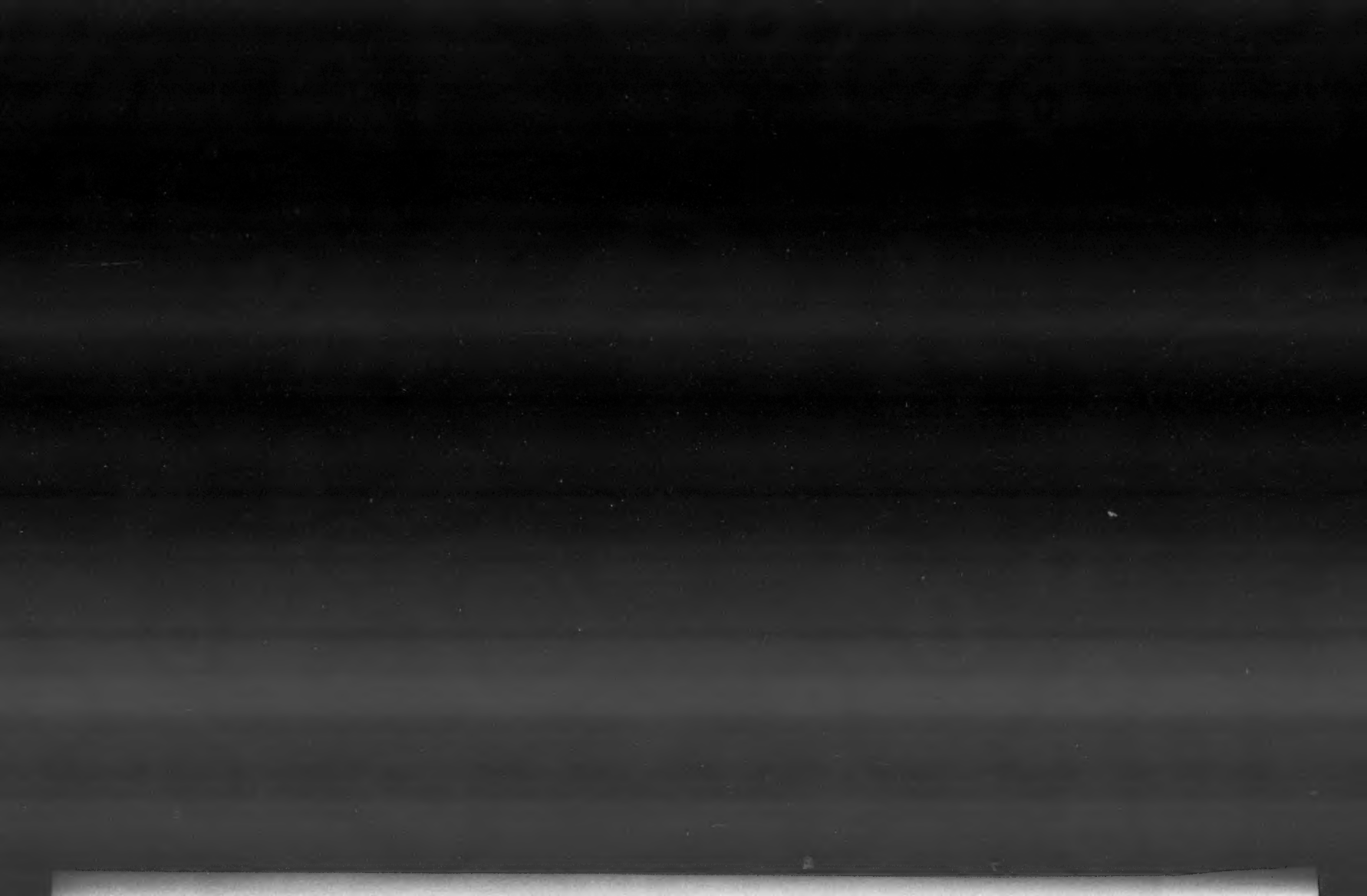
FIG. 7. A higher power of Fig. 6 shows relative preservation of peripheral optic nerve fibers. Spielmeyer stain. × 85.

FIG. 8. A higher power of Fig. 6 shows ballooning, fragmentation, and loss of myelin in the optic nerve. Spielmeyer stain. × 520.



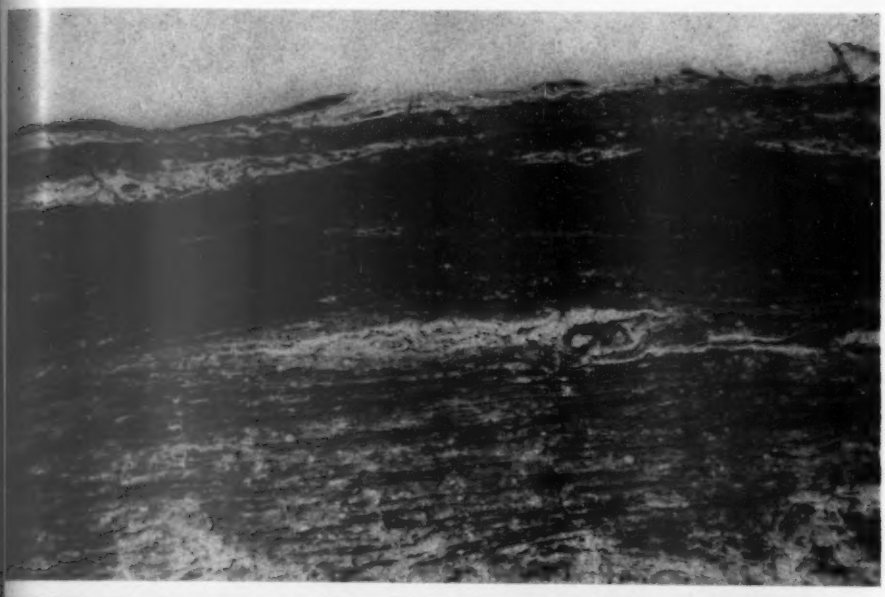


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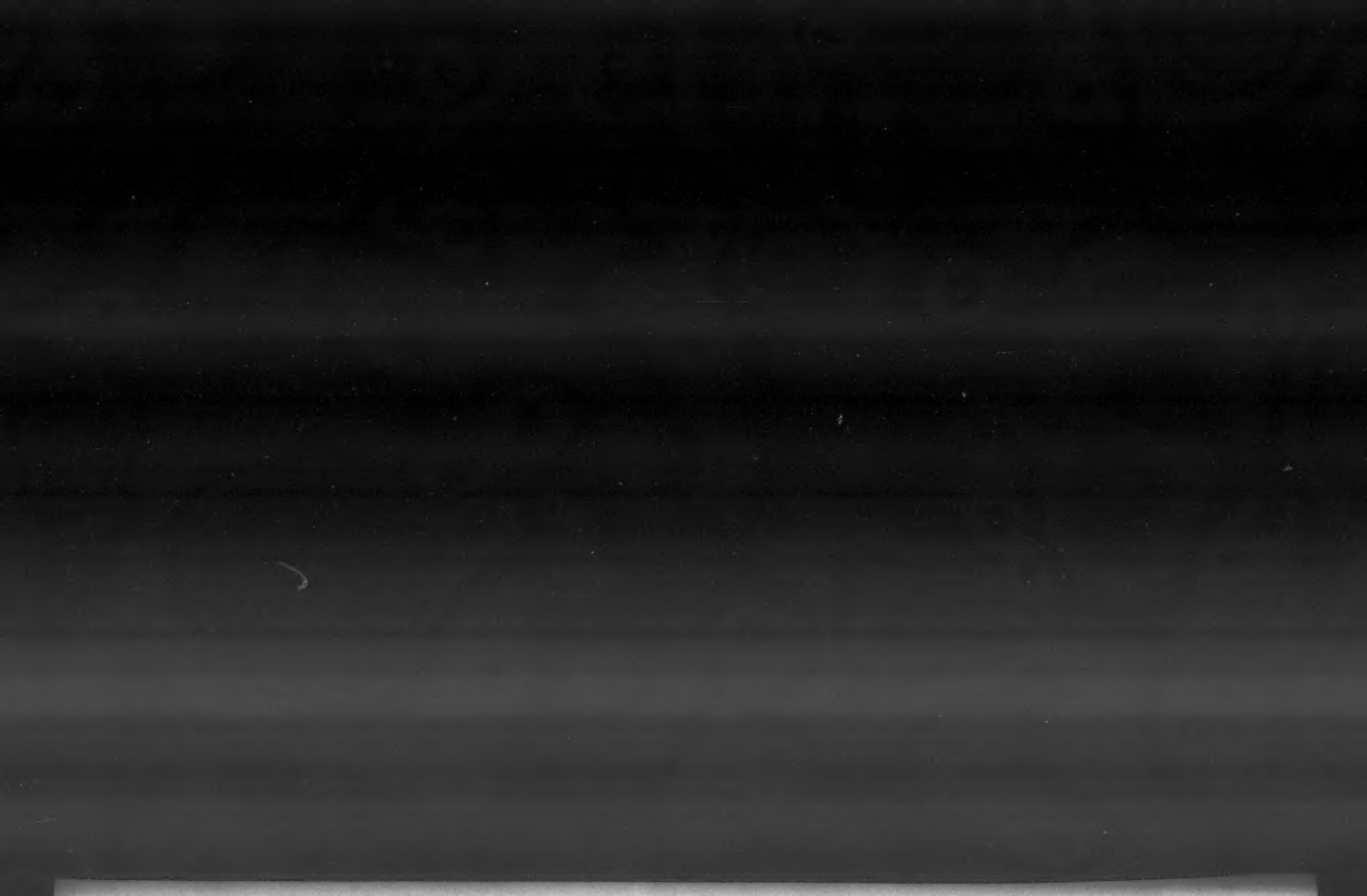
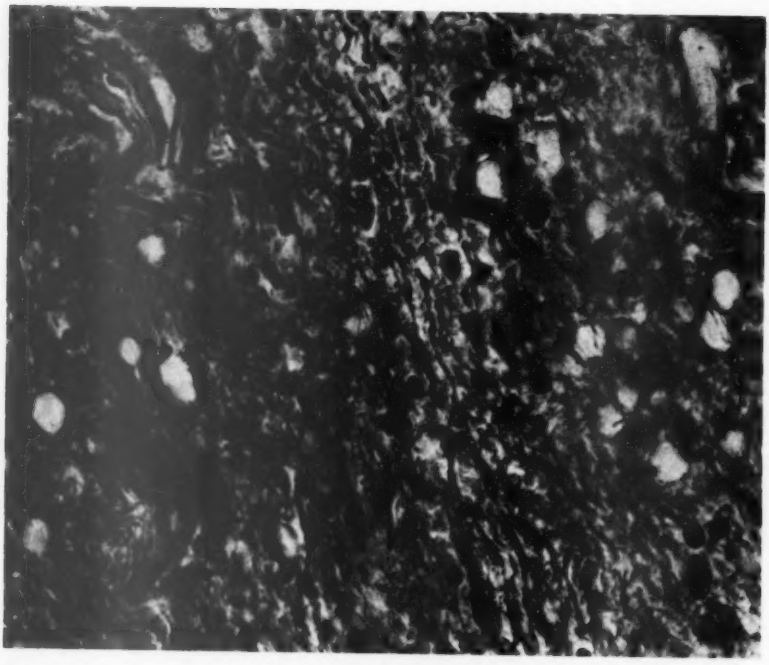
FIG. 9. The patient's optic nerve, showing swelling, fragmentation, and loss of axis cylinders. Bielschowsky stain. × 463.

FIG. 10. A normal geniculate body. Nissl stain. × 21.





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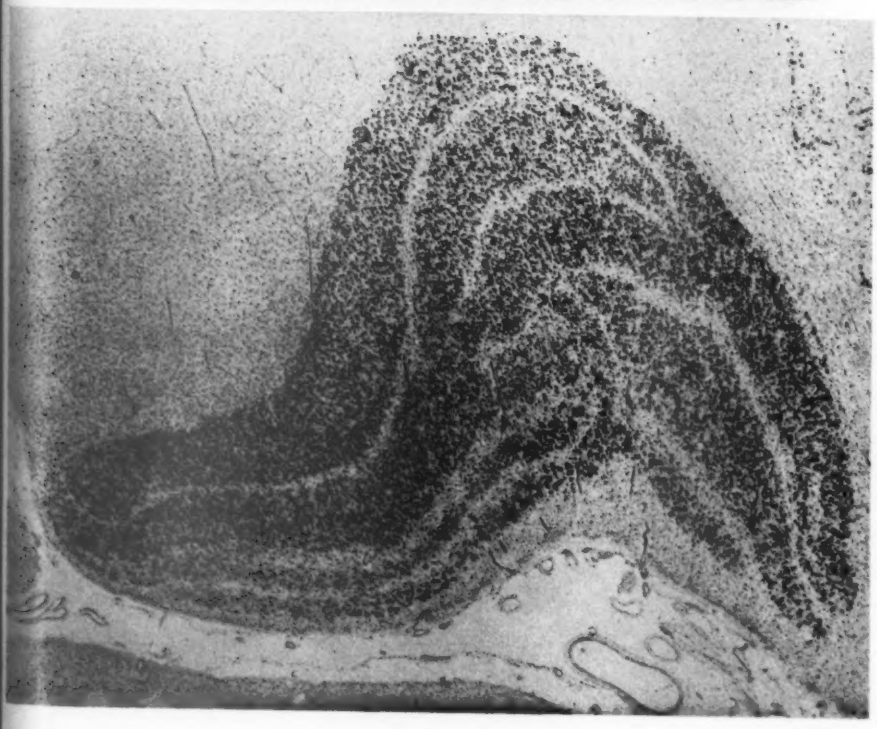

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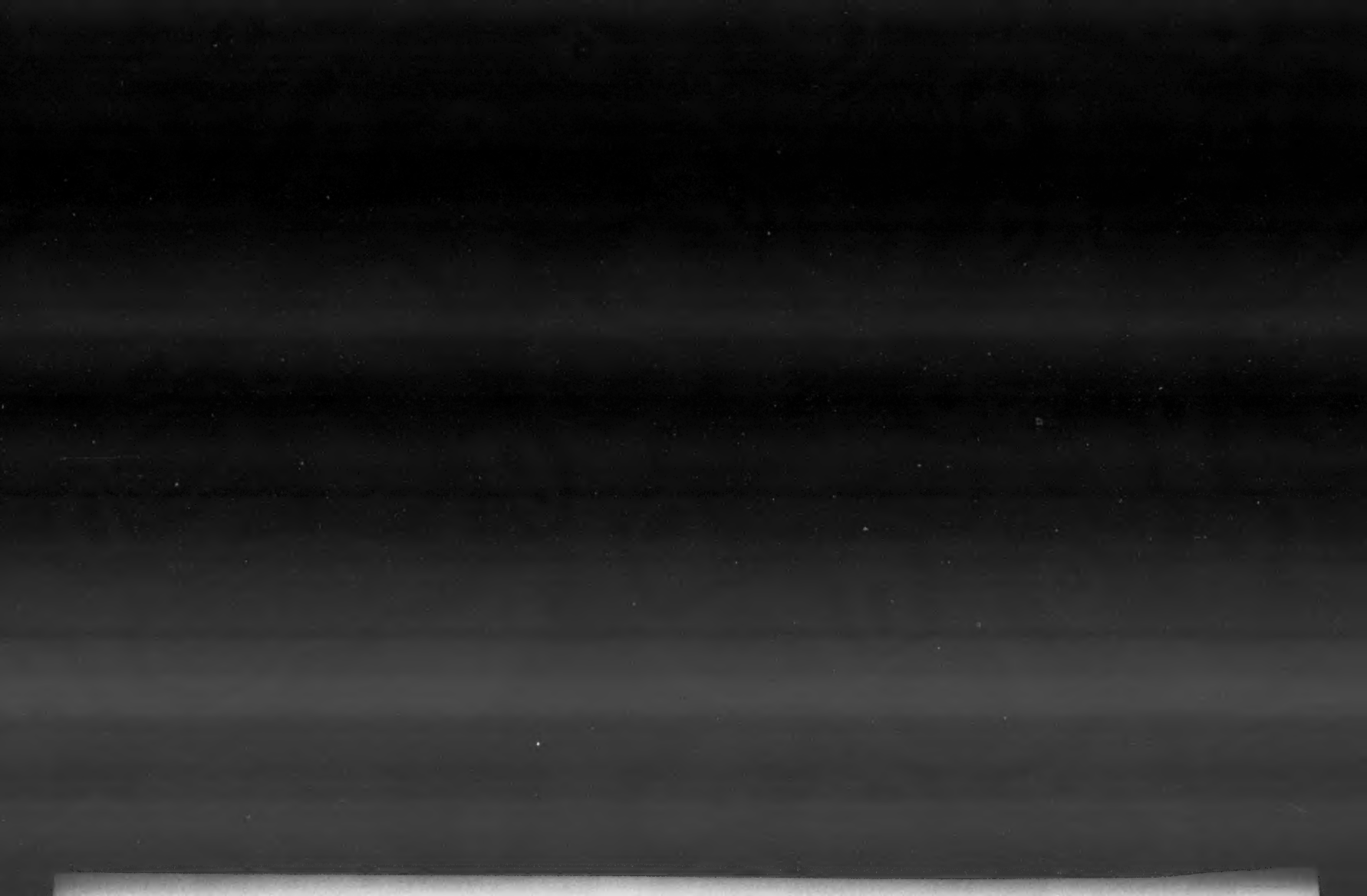
FIG. 11. The right geniculate body of the patient, showing cell atrophy, cell loss, and gliosis involving all 6 layers uniformly. Nissl stain. × 24.

FIG. 12. The left geniculate body of the patient, showing alterations similar to those in Fig. 11. Nissl stain. × 24.



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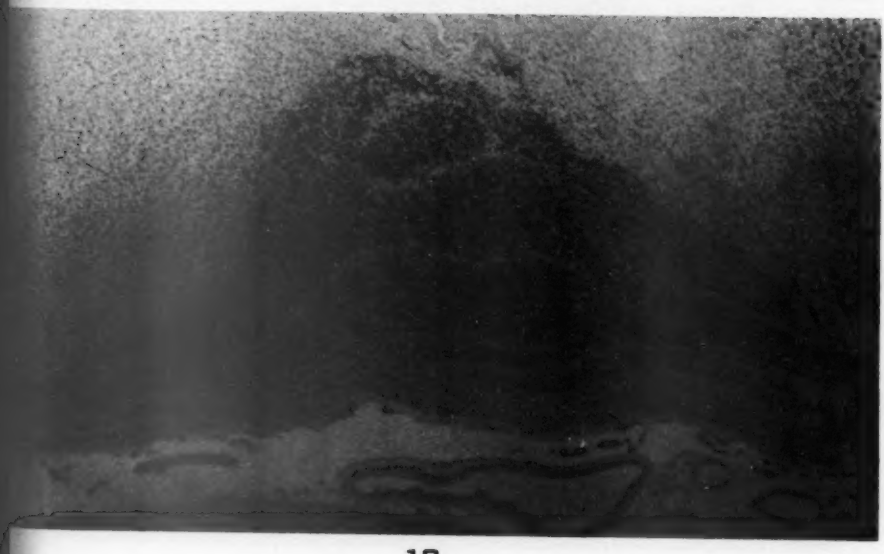

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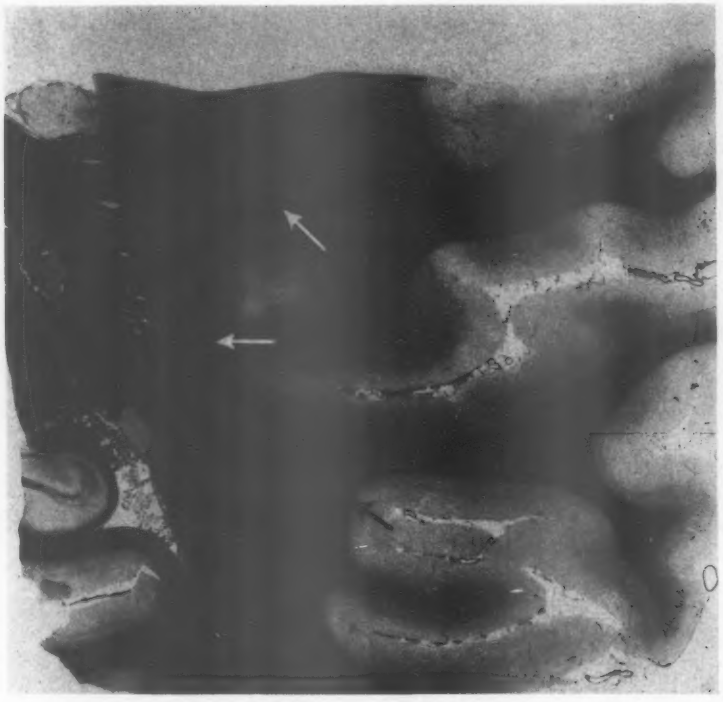
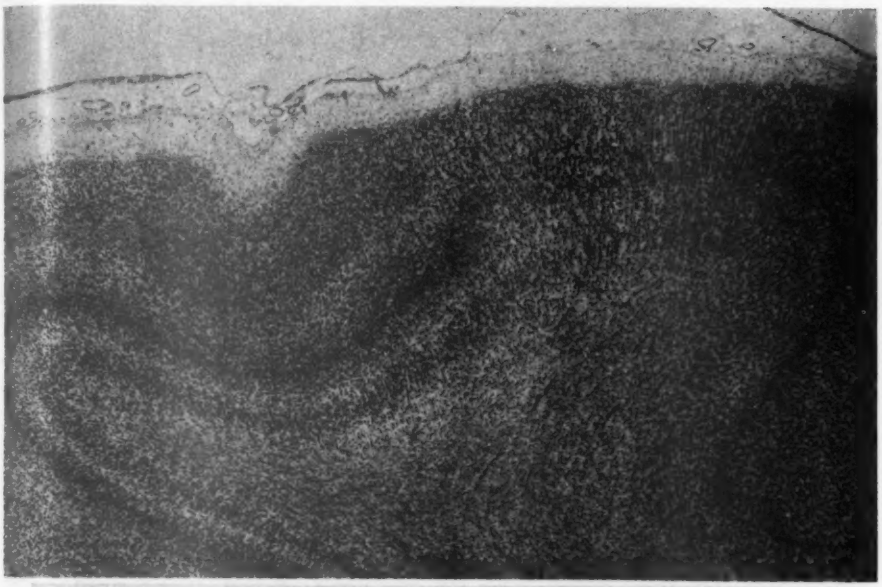


FIG. 13. The left temporal lobe of the patient, exhibiting loss of myelin in the geniculo-calcarine fibers, as shown by the arrows. Kultschitzky stain. × 2.

FIG. 14. The right occipital lobe of the patient, revealing a normal visual cortex. Nissl stain. × 25.

FIG. 15. The cervical portion of the patient's spinal cord, revealing demyelination of the gracilis columns. Weil stain. × 29.



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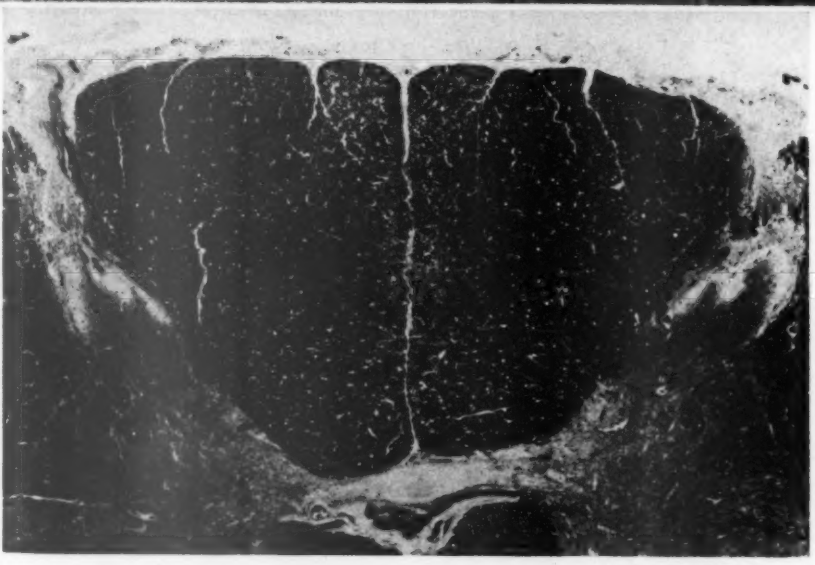

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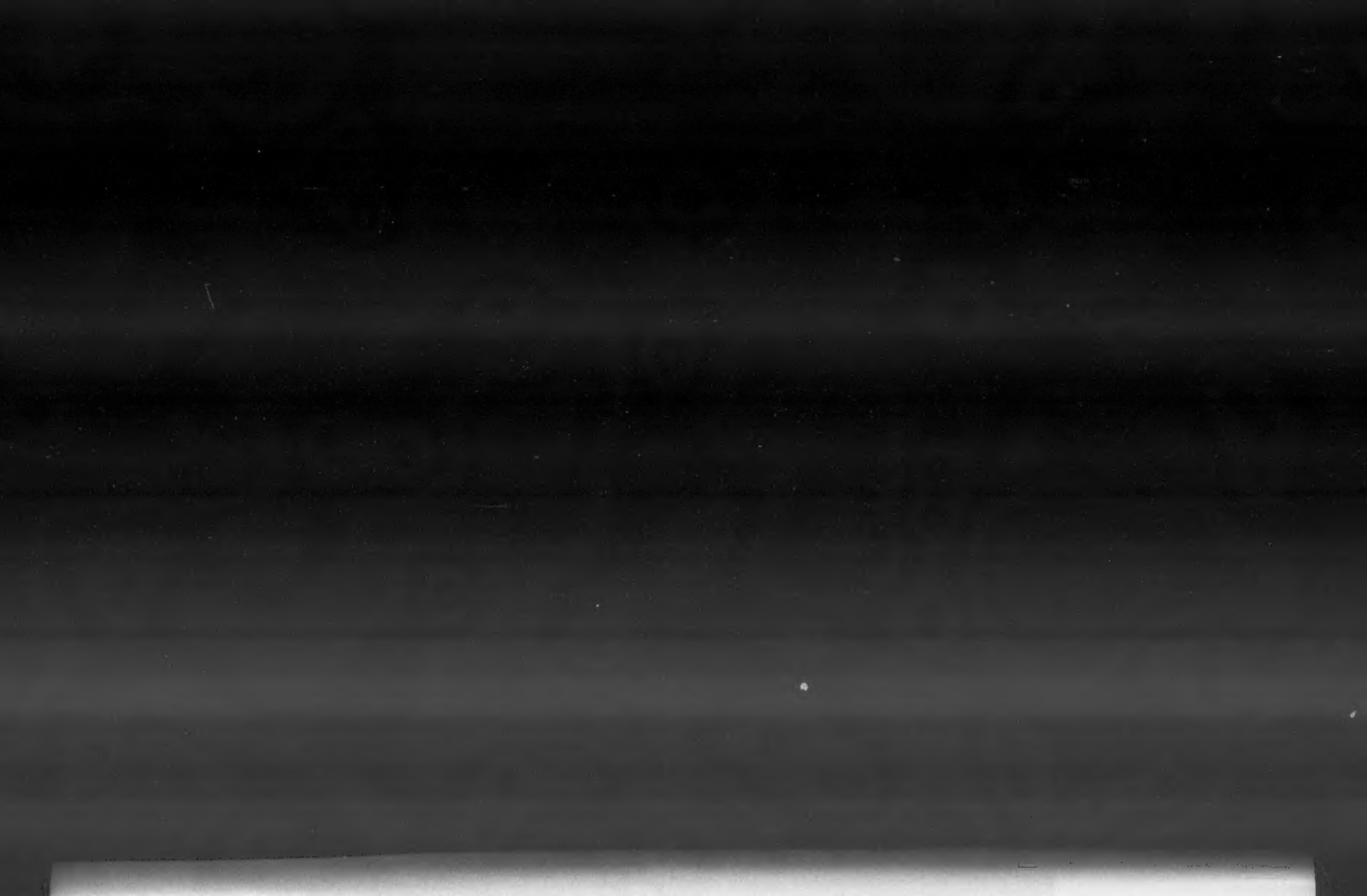
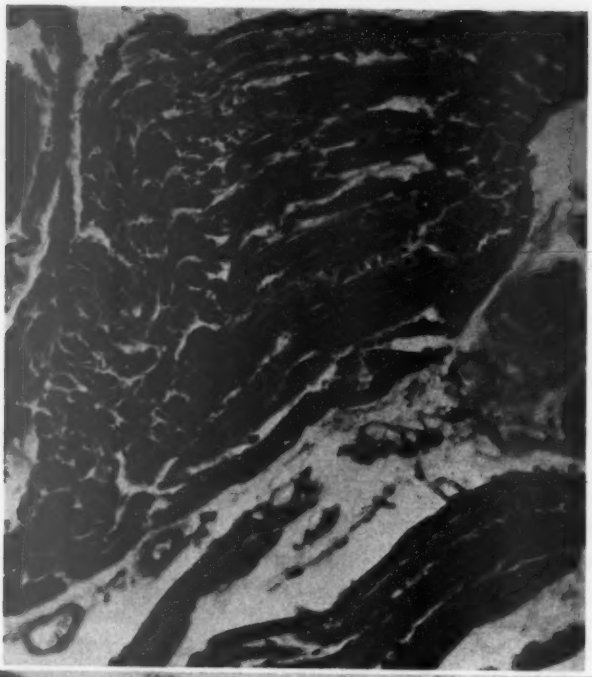
FIG. 16. The skeletal muscle supplied by the nerve in Fig. 17, demonstrating shrinkage of muscle fibers with sarcolemmal nuclear proliferation. Hematoxylin and eosin stain. × 200.

FIG. 17. The patient's popliteal nerve, showing ballooning, fragmentation, and loss of myelin. Spielmeyer stain. × 400.





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